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Changes in Intracellular Chloride During Osmotic Stress and L-alanine Uptake in Mouse Hepatocytes

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CHANGES IN INTRACELLULAR CHLORIDE DURING OSMOTIC STRESS AND L-ALANINE UPTAKE IN MOUSE HEPATOCYTES

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by

Kening Wang

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APPROVAL

This is to certify that the Graduate Committee of

Kening Wang

met on the

8th day of October, 1992.

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council and the Associate Vice-President for Research and Dean of the Graduate School, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Science.

Chairman, Graduate Committee

Signed on behalf of the Graduate Council

Associate Vice-President for Research and Dean of Graduate School.

ABSTRACT

CHANGES IN INTRACELLULAR CHLORIDE DURING OSMOTIC STRESS AND L-ALANINE UPTAKE IN MOUSE HEPATOCYTES

by

Kening Wang

A stable intracellular ionic environment is necessary for hepatocytes to function normally. Thus, during hypotonic shock or L-alanine uptake, hepatocytes swell and then exhibit a regulatory volume decrease (RVD), which comprises an increase in K^+ conductance (G_K), an increased K^+ efflux, and a hyperpolarization of transmembrane potential (V_m). Since hepatocyte intracellular Cl^- has been demonstrated to distribute passively with V_m, this study is designed to test the hypothesis that the hypotonic shock- or L-alanine uptake-induced hyperpolarization of V_m might provide an electromotive force for the efflux of hepatocyte intracellular Cl^-, which in turn would contribute osmotically to the RVD in hepatocytes. Double-barreled ion-selective microelectrodes were used to measure the changes of hepatocyte transmembrane potential, intracellular ionic activities [especially intracellular Cl^- activity, (a^i_{Cl})], and intracellular water volume during either anisotonic stress or L-alanine uptake. Hepatocyte V_m hyperpolarized, (a^i_{Cl}) decreased, intracellular K^+ activity (a^i_K) decreased, and intracellular water volume increased during hyposmotic stress. When perfused with L-alanine, hepatocyte V_m exhibited a transient depolarization followed by repolarization and then underwent a constant hyperpolarization. Meanwhile, hepatocyte intracellular Na^+ activity (a^i_{Na}) increased, a^i_K & a^i_{Cl} decreased, and intracellular water volume increased. In both hypotonic shock and L-alanine uptake conditions, the decreased a^i_K could be attributed to cell swelling, However, the decrease in a^i_{Cl} was greater than could be accounted for by cell swelling. When the change of V_m was inhibited by K^+ channel blockers, the change of a^i_{Cl} was also inhibited. Based on the measured a^i_{Cl}, Cl^- was always at its electrochemical equilibrium in all of the control and experimental conditions. The conclusions of this study emphasize the passive distribution of hepatocyte intracellular Cl^- with the changes of V_m induced by hypotonic stress and L-alanine uptake. Thus, the data strongly support the idea that the hypotonic shock- or L-alanine uptake-induced hyperpolarization of V_m provides electromotive force for the efflux of hepatocyte intracellular Cl^-. This could contribute to hepatocyte volume regulation during both hypotonic shock and organic solute transport.

DEDICATION

I dedicate this dissertation to my wife and my parents for their love and encouragement and to my aunt for her support during my studies in the United States.

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CONTENTS

CHAPTER Page

LIST OF FIGURES

Figure Page

LIST OF TABLES

CHAPTER 1

GENERAL INTRODUCTION

The Purpose and the Significance of this Study

Stable intracellular ionic activity, pH, and metabolic enzyme concentration are necessary for hepatocytes to function normally. Unless attenuated by certain compensatory mechanisms, any change in cell volume will interrupt the intracellular environmental balance and, thus, interfere with normal hepatocyte function. Hepatocyte edema also causes hepatomegaly. This can compress the circulation and cause an increase in microcirculatory resistance. Recent studies have shown, in isolated perfused rat liver, that hypotonic perfusate results in cell swelling, stimulates a regulatory volume decrease (RVD) process, and induces an increase of perfusion pressure (Bruck, R., P. Haddad, J. Graf, and J. L. Boyer, 1992). The increased perfusion pressure is consistent with persistent sinusoidal compression, which would result from the more sustained liver cell swelling. Hepatocyte enlargement due, in part, to excess water retention has been proposed as a primary cause of portal hypertension in alcohol-fed rats and in patients with alcoholic cirrhosis (Bruck, R., P. Haddad, J. Graf, and J. L. Boyer, 1992).

The term regulatory volume decrease (RVD) refers to the process of recovery of cells toward their original volume

from hypotonic swelling or from swelling induced by other biological stimuli such as glucose or amino acid transport. The RVD mechanism in hepatocytes comprises alterations in various biophysical and biochemical processes. A hyperpolarized transmembrane potential (V_m), an increased K^+ and Cl^- conductance (G_K and G_{Cl}), and hence an increased K^+ efflux are involved in the RVD process at the level of hepatocyte plasma membrane. More details about the mechanisms of RVD in hepatocytes will be introduced later in this chapter.

The hypothesis of this experiment is that hypotonic shock or L-alanine uptake-induced hyperpolarization of V_m may provide an electromotive force for the efflux of hepatocyte intracellular Cl^-, which in turn contributes osmotically to the RVD in hepatocytes. Hepatocyte V_m has been demonstrated to hyperpolarize due to an increase in K^+ conductance during hypotonic shock (Howard, and Wondergem, 1987) and prolonged L-alanine uptake (Kristensen, 1986) and Cl^- has been found to distribute passively across the plasma membrane with V_m in hepatocytes (Lyall, Croxton, and Armstrong, 1987). Successful completion of these studies will provide evidence to link these two events together with the physiologically important cellular function--cell volume regulation. Any findings from this experiment will have broad implications for our understanding of hepatocyte function and pathology.

The Mechanism of Cell Volume Regulation in Hepatocyte

Cell volume is determined largely by cell water content. Cellular water content, in turn, depends on the solute content of the cell and, in particular, on the amount of the most abundant permeable intracellular inorganic ions: K^+, Na^+, and Cl^-. Thus animal cells have been found to regulate their volume by adjusting their intracellular ion content (Haddad, Thalhammer, and Graf, 1989). In general, volume regulation implies the maintenance of constant cell volume under isotonic conditions, or controlled return of shrunken or enlarged cells toward their original volume. The "pump and leak" model was established more than 50 years ago to explain the mechanism of maintaining constant cell volume in isotonic condition. It was proposed that a stable cell volume depends on the balance between "pump"-mediated active transport of Na^+ and K^+ and "leaks" of Na^+ and K^+ down their respective electrochemical gradients. In cases of anisotonic conditions, regulatory volume increase (RVI) and regulatory volume decrease (RVD) are the terms used to describe the process by which cells return toward their original volume from shrunken and swollen states, respectively.

In most vertebrate cells RVD is mediated to a large extent by passive KCl loss through parallel K^+ and Cl^- channels, parallel K^+-H^+ and Cl^--HCO_3^- exchangers or coupled KCl cotransporters (Chamberlin, and Strange, 1989). RVI in

most vertebrate cells studied so far is mediated primarily by one of the two mechanisms. Volume regulatory Na^+-K^+-$2Cl^-$ cotransport pathways have been described in avian red blood cells, frog skin, Ehrlich ascites tumor cells, and Madin-Darby canine kidney (MDCK) cells. Parallel Na^+-H^+ and Cl^--HCO_3^- exchangers mediate RVI in human lymphocytes, Necturus gallbladder, and mouse medullary thick ascending limb. A NaCl cotransporter activated after cell shrinkage has also been found in Ehrlich cells (Chamberlin, and Strange, 1989).

In addition to adjusting their intracellular ion content as the way to regulate cellular volume, many organisms and cell types, such as bacteria, invertebrates, algae, and Ehrlich ascites, utilize both inorganic ions and organic compounds for volume maintenance. It is suggested that in these organisms available inorganic ions mediate short-term volume regulation but that long-term volume maintenance depends on the accumulation of more compatible organic solutes (Chamberlin, and Strange, 1989). These phenomena are likely to occur in many organisms and cell types, including cells of the mammalian renal medulla.

Another possible mechanism that may contribute to the cell volume regulation is the cytoskeleton. In red blood cells, actin is linked to the cell membrane by spectrin and other proteins. Spectrin makes the linkage to the membrane by association with ankyrin, which in turn binds to band 3 protein, known also as the Cl^--HCO_3^- exchanger. Research

evidence suggests that the cytoskeleton system may control cell volume through the following ways: 1) The actin and actin associated protein are potentially contractile. Through a connection with cell membrane, the contractile apparatus could exert enough tension to act as a resistive force that inhibits swelling of the cell (Heubusch, Hung, and Green, 1984; Mills, 1987). 2) Cytoskeletal protein might interact with other membrane proteins, and thereby control the activity of these membrane proteins. These membrane proteins could include ion channels, pumps, and ion exchangers, such as the band 3 anion transporter (Mills, 1987). 3) Certain evidence suggested that the cytoskeletal system is related to the vesicle insertion/retrieval process. This may contribute to volume regulation directly by dumping contents of the vesicle into the lumen or other extracellular space (Van Rossum, Russo, and Schisselbauer, 1987). The indirect way this works for controlling the cell volume is that the membrane of the vesicle may contain ion and water channels. The fusion of the vesicle membrane with the cell membrane could insert those channels to the cell membrane (Mills, 1987). A vesicle insertion mechanism for insertion of water channels into the apical membrane of toad bladder epithelial cells, proton pumps in turtle urinary bladder, and KCl pathways in Necturus gallbladder have been proposed during volume regulation (Mills, 1987). A microfilament mediated vesicle insertion mechanism that

would deliver preformed Na^+ channels to the apical membrane has also been proposed for rabbit urinary bladder (Loo, Lewis, Ifshin, and Diamond, 1983). In rat liver, cells swell during incubation at 1°C. Upon rewarming, fluid-filled vesicles appear and are expelled via exocytosis. The recovery of volume, but not the appearance of fluid-filled vesicles, was inhibited by cytochalasin B. Thus, the role of actin filaments in this case may be related to "shuttling" the vesicle to the cell membrane, which contains fluid destined to be expelled (Mills, J. W., 1987).

The normal functioning of hepatocytes demands a stable intracellular ionic and pH environment. This requires hepatocytes to regulate their volume even when the extracellular osmolality remains constant, although this is not always the case because the intestinal absorption usually changes the osmolality of the portal blood. Moreover, the processes of membrane transport, cellular metabolism and bile secretion in hepatocytes constantly change the content and concentration of intracellular organic solutes, which are accompanied by the movements of water and inorganic ions across the cell membrane. Examples include hepatocyte intracellular accumulation of amino acids through a Na^+-amino acid cotransport system and hormone stimulated intracellular glycogenolysis.

In hepatocytes, relatively little is known about RVI. Although there is indirect evidence suggesting the possible

existence of the RVI mechanisms in hepatocytes (Haddad, Thalhammer, and Graf., 1989), most studies favor the notion that hepatocytes lack the RVI mechanisms by showing that 1) hepatocytes kept their shrunken volume after hypertonic shock for at least 15 min (Bakker-Grunwald, 1983). 2) most common RVI mechanisms such as Na^+-K^+-2Cl^- or Na^+-Cl^- cotransporters and parallel Na^+-H^+, Cl^--HCO_3^- exchangers were not found in the basolateral membrane of hepatocyte (Nathanson, and Boyer, 1991).

In contrast with RVI, the RVD mechanisms have been demonstrated to exist in hepatocytes when they are challenged by hypotonic stress or when they take up amino acids. This RVD process is accompanied by an increased K^+ permeability of the cell membrane and by an increased K^+ efflux, which is sensitive to barium and quinine. Since it is generally assumed that macroscopic electroneutrality is preserved during RVD, the same amount of anions are expected to exit the cell. (Grinstein, Clarke, Dupre, and Rothstein, 1982). In liver, the best candidate is Cl^-, because Cl^- has been demonstrated to be passively distributed (Lyall, Croxton, and Armstrong, 1987) and the Cl^- permeability of hepatocyte membrane is very high (Moule, Bradfold, and McGivan, 1987).

Hepatocyte V_m hyperpolarizes with hypotonic shock (Howard, and Wondergem, 1987) and during prolonged alanine uptake (Wondergem, and Castillo, 1988). A more negative

intracellular environment will certainly facilitate the efflux of Cl^-. However, compared with the cation fluxes, relatively little is known about the role of intracellular anions in the mechanism of volume regulation in hepatocytes. Corasanti et al. (1990) recently evaluated the critical role for intracellular Cl^- in the RVD of rat hepatocytes. They showed that following exposure to hypotonic medium, isolated rat hepatocytes swelled as osmometers within 30-60 s and subsequently underwent regulatory volume decrease back toward the control volume. Either high extracellular K^+ concentration, barium, or quinine blocked this volume recovery. Cl^- depletion inhibited RVD by 40% while 0.5 mM 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) blocked the recovery by almost 90%. Their findings suggest that quinine- and barium-sensitive K^+ conductance and DIDS-sensitive anion conductance, which is partly accounted for by Cl^-, mediate RVD in rat hepatocytes (Corasanti, Gleeson, and Boyer, 1990). A recent report from Haddad et al. (Haddad, Beck, Boyer, and Graf, 1991) showed that a step decrease in external Cl^- accelerated the initial rate of RVD more than twofold; whereas, depleting the cells of Cl^- abolished RVD. Although there is a disagreement between these studies on the relative percentage of inhibition of RVD in Cl^--depleted hepatocytes, their results have certainly confirmed the role for Cl^- in the hepatocyte RVD mechanism.

Cl^- is a critical component of RVD mechanisms of many cell types. In the Necturus gallbladder, removal of Cl^- from bathing solution completely blocks the RVD response induced by hypotonic stress (Larson, and Spring, 1984). In frog skin epithelium, hypotonic stress induced a increase in the basolateral Cl^- permeability. This increase in Cl^- permeability possibly occurs in combination with an increase in the permeability to K^+ of the basolateral membrane. Cl^- will thus leave the cell together with K^+ (Larson, M., and K. R. Spring, 1987). In lymphocytes, a marked increased efflux of Cl^- due to the increase in Cl^- permeability in hypotonically swollen cells has been reported. Although K^+ permeability is also increased by hypotonic cell swelling, their conductances seem to be independent of each other in the process of RVD (Grinstein, Clarke, Dupre, and Rothstein, 1982).

The intracellular signaling for activating RVD mechanisms in hepatocyte is still unclear. Hepatocyte swelling is believed to be the initial signal for the activation of RVD in both hypotonic shock and alanine uptake conditions (Bakker-Grunwald, 1983). Cell swelling could directly activate stretch-activated K^+, Na^+ or Cl^- channels in the membrane. Volume-regulatory K^+ movements have been characterized in liver perfused with hypotonic or hypertonic media, and it seems likely that such ion fluxes occur via stretch-sensitive K^+ channels (Lang, et al., 1989). Several

studies have demonstrated the activation of Ba^{2+} and quinine-sensitive K^+ channels, which mediate RVD after hypotonic shock (Moule, and McGivan, 1990). Some studies suggest that Ca^{2+} could mediate RVD in hepatocytes (Bear, and Petersen, 1987; Bear, 1990; Khalbuss, and Wondergem, 1991). A cell swelling induced transient activation of a stretch-activated nonselective cation channel was reported in rat hepatocytes (Bear, 1990). This channel was permeable to Na^+, K^+, and Ca^+ and was proposed to be responsible for the hypotonic shock induced elevation of intracellular Ca^{2+} observed in their experiments. Since Ca^{2+}-dependent K^+ channel has been also shown to be present in hepatocytes, it was suggested that Ca^{2+} influx through the nonselective cation channel may act as the primary signal of cell swelling, which in turn activates Ca^{2+}-dependent K^+ channel effective in cell volume regulation (Bear, 1990). Rising cytoplasmic free Ca^{2+} concentration activates K^+ and Cl^- conductance in guinea-pig hepatocytes (Capiod, 1987). Other factors that might be involved in hepatocyte RVD mechanisms include intracellular pH and cAMP, because they have been shown to influence membrane K^+ permeability in hepatocytes (Moule, and McGivan, 1990).

The following model of RVD during hypotonic shock in hepatocyte has been proposed: Hypotonic shock causes an increase in intracellular water content, which induces hepatocyte swelling. Cell swelling stimulates the increase

in K^+ and Cl^- conductance, which may be mediated by increase of intracellular Ca^{2+}. The increased K^+ conductance causes an increase in K^+ efflux, which causes the hyperpolarization of the cell membrane. This hyperpolarization induces an increase in efflux of intracellular Cl^-. Both K^+ and Cl^- efflux cause cell volume recovery.

There may be other RVD mechanisms present in hepatocytes. One of the major functions of liver is bile secretion. Recently, Bruck et al. (1992) reported that RVD stimulates bile flow, bile acid excretion, and exocytosis in isolated perfused rat liver. Their studies suggest that microtubule-dependent vesicular excretory mechanisms are involved in hypotonically induced choleresis and the stimulation of bile acid and HRP (horseradish peroxidase), a fluid phase marker, excretion. Exocytosis of pericanalicular vesicles could serve both to deliver fluid into the lumen and to insert membrane transporters into the cell membrane domain. Both Cl^- and K^+ channels have been identified in isolated rat canalicular membranes. Quantitatively, water efflux into bile represents a minor fraction of total water efflux from the liver during RVD, probably reflecting the relative small surface area of the canalicular membrane of the hepatocyte. Their studies demonstrated, however, that the canalicular bile secretion is stimulated by RVD through mechanisms that involve K^+ channels, bile acid transport, and microtubule-dependent

vesicular transport (Bruck, Haddad, Graf, and Boyer, 1992).

L-alanine Uptake in Hepatocytes and the Response of Hepatocyte V_m, a^i_K, a^i_{Cl}, and Cell Water Volume During L-alanine Uptake

Alanine occupies a key position in amino acid metabolism of the mammalian organism. It accounts for over 50% of total hepatic amino acid uptake (Felig, 1975). In the glucose-alanine cycle, alanine is synthesized in muscle by transamination of glucose-derived pyruvate, released into the bloodstream, and taken up by liver. In liver, the amine group of alanine is used for urea synthesis and the carbon skeleton of alanine is reconverted to glucose and released into the blood stream, where it is available to muscle for resynthesis of alanine. Thus, alanine serves to transfer nitrogen to the liver for urea synthesis, and simultaneously to supply carbon atoms for hepatic gluconeogenesis (Felig, 1975; Felif, Pozefsky, Marliss, and Cahill, 1970; Kristensen, 1986). Since the alanine transport across the liver cell membrane seems to be a regulated, key process in amino acid metabolism of the body, it has attracted considerable interest for many years.

It has well been established that the majority of alanine influx in hepatocytes is Na^+ dependent and is stimulated by intracellular negativity. The ratio between cotransported Na^+ and alanine is 1 : 1 (Kristensen, 1986).

The transmembrane Na^+ electrochemical gradient seems to be the exclusive driving force for intracellular alanine accumulation. At least eight distinct amino acid transport systems have been identified in hepatocytes. System A is the one that accounts for most of alanine uptake (Kilberg, 1982; Wondergem, and Castillo, 1988).

The influx of alanine across the liver cell membrane occurs through both Na^+-dependent and Na^+-independent pathways. At low extracellular alanine concentrations the Na^+-dependent influx is by far the most important. The concentration of alanine in portal blood plasma of the rat varies in the range from 0.2 to 2 mM, depending on the physiological and nutritional conditions (Kristensen, 1986). At such alanine concentrations the contribution by Na^+-dependent alanine influx in isolated rat hepatocytes amounts to 80-95% of the total influx. Evidence has shown that alanine transport occurs exclusively in the blood-sinusoidal membrane of the hepatocytes (Kristensen, 1986). Actually, Na^+-dependent alanine influx in hepatocyte is mediated by two separate transport systems, A and ASC, with broad specificities towards different neutral amino acids. At a physiological extracellular alanine concentration and ion composition the majority (~70%) of the Na^+-dependent alanine influx is mediated by system A. This transport system has been shown to respond to a variety of physiological stimuli. Prior fasting of the animals, protein-rich diet, and certain

hormones such as catecholamines, glucagon, glucocorticoids, growth hormone, insulin, and thyroid hormones are all known to stimulate transport system A (Kristensen, 1986; Kilberg, 1982).

The Na^+-coupled alanine influx in hepatocytes is accompanied by changes in various other transport processes. A transient depolarization followed by sustained hyperpolarization of the cell membrane was observed (Kristensen, and Folke, 1983; Wondergem, and Castillo, 1988). The hepatic alanine uptake capability is extremely high. At a physiological transmembrane Na^+ gradient, the intracellular alanine can exceed the extracellular concentration about 20-fold, but metabolism will exert a conspicuous sink effect. After addition of 10 mM alanine to the extracellular medium, an intracellular alanine accumulation of 93 mM was observed when the alanine metabolism was inhibited in isolated rat hepatocytes (Kristensen, 1986). Simultaneously, the cellular Na^+ concentration increases by 10 mM within a few minutes; the cellular K^+ concentration decreases by 38 mM during 10 to 20 min and cellular water volume increases to 112% of the control value (Kristensen, 1986). The alanine induced increase in K^+ permeability and K^+ efflux was proposed to compensate the cell volume change and is responsible for the hyperpolarization of the hepatocyte membrane (Bear, 1990; Kristensen, 1980; Wondergem, and Castillo, 1988). Alanine

uptake also stimulates the Na^+/K^+ pump. This was estimated from the initial rate of ouabain-sensitive ^{42}K uptake and could be the result of stimulating effect of increased intracellular Na^+ (Kristensen, 1986). Relatively little is known about the response of hepatocyte intracellular Cl^- to alanine uptake. However, an inverse correlation between the Cl^- distribution (expressed as the ratio of internal to external concentrations) and the initial rate of alanine transport has been reported by Moule and colleagues (1987).

The general model for hepatocyte alanine uptake is as follows: Na^+-coupled uptake of alanine increases intracellular Na^+, which leads to an increase in active Na^+-K^+ pumping and thus in K^+ influx; influx of alanine and cations tends to increase the cellular content of osmotically active substances implying a tendency to water uptake; cell swelling induces an increase in the permeability of a conductive pathway for leading to net efflux of K^+ (with accompanying anions) and cellular hyperpolarization. Net efflux of K^+ prevents excessive cell volume increase during amino acid accumulation, whereas hyperpolarization tends to support the driving force for alanine influx and anion efflux. The pathway for K^+ efflux is most likely a K^+ channel (Kristensen, 1986).

The Response of Hepatocyte V_m, a^i_K, a^i_{Cl}, and Cell Water Volume During Osmotic Stress

Studies from our laboratory have already shown that hepatocytes respond to the changes of extracellular osmolality by changing their V_m, a^i_K, and intracellular water volume. During hyperosmotic stresses, the V_m depolarizes, a^i_K increases, and the hepatocytes shrink (intracellular water volume decrease) (Wang, and Wondergem, 1991; Khalbuss, and Wondergem, 1990; Bakker-Grunwald, 1983). The depolarization of V_m in this case was unexpected since the increased a^i_K will produce a greater transmembrane K^+ electrochemical gradient, which should cause a hyperpolarization based on the Nernst equation. But further studies indicate that hepatocyte membrane K^+ conductance, G_K, decreases during hypertonic shock and this at least partially accounts for the depolarized V_m (Wang, and Wondergem, 1991). The increased a^i_K is most likely due to the shrinkage of the cell, although reducing K^+ efflux caused by a decrease in K^+ permeability and activation of Na^+/K^+ pump may also contribute to the increased a^i_K. The fact that hepatocytes shrink during hyperosmotic stress has been confirmed in different laboratories (Bakker-Grunwald, 1983; Khalbuss and Wondergem, 1990; Wang and Wondergem, 1991).

It is not difficult to imagine that during hypotonic shock, hepatocyte V_m, a^i_K, and intracellular water volume

behave just the opposite as they do during hyperosmotic stress. Hepatocyte V_m hyperpolarizes, a^i_K decreases, and hepatocytes swell during hypotonic shock (Howard and Wondergem, 1987; Khalbuss and Wondergem, 1990; Cohen and Lechene, 1990). Several studies have suggested that an increased membrane K^+ conductance during hypotonic stress is responsible for the hyperpolarization of V_m (Howard and Wondergem, 1987; Khalbuss and Wondergem, 1990). Several other studies also suggested indirectly that hepatocytes lose intracellular K^+ during hyposmotic stress, and this could be true especially during prolonged hypotonic stress. By monitoring the effluent K^+ activity, Haddad et al. have shown that initiation and termination of hypotonic stress in isolated perfused rat liver triggered a sharp transient net water movement into and out of the liver. In addition, hypotonic stress also caused a large transient net release of hepatic K^+ which is sensitive to barium and quinine (Haddad and Graf, 1989). Using the same methods, Haussinger et al. (1990) reported similar results. Their data suggest an increase in G_K and a net loss of intracellular K^+ during hypotonic shock induced RVD in hepatocytes. Using the electron probe microanalysis technique, Brian J. Cohen et al. (1990) found hypotonic solution led to a significant net loss of K^+ in primary cultured hepatocytes.

Isolated hepatocytes present a regulatory volume decrease process during hypotonic shock. When exposed to

hyposmotic medium, hepatocyte swelling was followed by shrinking toward the original volume (Corasanti, Gleeson, and Boyer, 1990; Bakker-Grunwald, 1983). In both isolated hepatocytes (Corasanti, Gleeson, and Boyer, 1990) and liver slices (Khalbuss, and Wondergem, 1990), the increased cell water volume is less than that expected if the cell responded as a perfect osmometer. All these imply the existence of an RVD mechanism in hepatocytes.

It is known that hepatocytes face osmotic challenges under physiological conditions. Intestinal absorption usually changes the osmolality of the portal blood and the process of membrane transport, cellular metabolism and bile secretion in hepatocytes constantly change the content and concentration of intracellular organic solutes. It is also known that these events are accompanied by the movements of water and inorganic ions across the cell membrane. From this point of view, the behavior of hepatocyte V_m, a^i_K, and a^i_{Cl} during osmotic stresses should have their physiological functions. Since change in hepatocyte volume is an obvious consequence after osmotic stresses, it is reasonable to relate these ionic events with the mechanism of hepatocyte volume regulation. Actually, decrease in hepatocyte a^i_K during hyposmotic stress has been suggested to be part of the regulatory volume decrease mechanism (Haddad and Graf, 1989) and a^i_{Cl} has also been suggested to be important for the cell volume recovery after hypotonic shock in isolated

rat hepatocytes (Carosanti, Gleeson, and Boyer, 1990; Haddad, Beck, Boyer, and Graf, 1991).

The Transmembrane Potential (V_m) of Hepatocyte

1) The composition of V_m in hepatocyte:

In normal physiological conditions, the transmembrane potential of hepatocytes, V_m, in rat or mouse range from -30 to -40 mV (Moule and McGivan, 1990). This observed V_m value is clearly much less than the K^+ equilibrium potential, which is about -80 to -88 mV in hepatocytes (Graf, Haddad, Haussinger, and Lang, 1988; Moule and McGivan, 1990; Wondergem, and Castillo, 1986). So, based on the ratio of the K^+ concentration difference across the membrane, it is clear that the V_m of hepatocytes, unlike that in excitable cells, is not simply a consequence of the K^+ electrochemical potential gradient. Actually, hepatocytes have a relatively low electrogenic permeability to K^+ ions under normal conditions (Moule and McGivan, 1990; Wang and Wondergem, 1991). So any factor that increases K^+ conductance, G_K, of the hepatocyte membrane will cause hyperpolarization of the V_m. The hyperpolarizing effect of adrenergic hormone or glucagon on hepatocyte V_m is a good example of this aspect (Moule and McGivan, 1990; Graf and Petersen, 1978).

Another factor that may account for the hepatocyte V_m being lower than the K^+ equilibrium potential is leakage of

Na^+ from outside to inside the cell. As in other non-excitable cells, the low membrane potential of hepatocytes has been attributed to a relatively high Na^+ permeability of the cell membrane (Graf, and Petersen, 1978).

Cl^- has been demonstrated to distribute passively in hepatocytes (Lyall, Croxton, and Armstrong, 1987). This finding implies that the permeability of the cell membrane to Cl^- is much higher than that of other ions, including K^+ (Moule and McGivan, 1990). Some measurements have shown that the Cl^- conductance, G_{Cl}, is higher than that of K^+ in hepatocytes (Graf and Petersen, 1978).

Hepatocytes contain an active Na^+/K^+ ATPase which is located on the sinusoidal and lateral plasma membrane (Nathanson and Boyer, 1991; Moule and McGivan, 1990). This ATPase exchanges three intracellular Na^+ ions for two extracellular K^+ ions, establishing chemical gradients for K^+ and Na^+ across the basolateral membrane. In addition, this cation pump generates a negative electrical potential intracellularly that functions as an electrogenic driving force for secondary and tertiary active transporters (Nathanson and Boyer, 1991). The pump is sensitive to intracellular Na^+. The estimated K_m for Na^+ of the Na^+/K^+ pump (10-37 mM) encompass the range (7-22 mM) of intracellular Na^+ concentration in liver (Moule and McGivan, 1990). Although it is frequently stated that the immediate quantitative contribution of the Na^+/K^+ pump activity to the

membrane potential is small (Graf, Haddad, Haussinger, and Lang, 1988), some experiments still suggest that this pump activity may be a major determinant of hepatocyte transmembrane potential (Moule, and McGivan, 1990).

Other factors that may play a role in determination of hepatocyte V_m include $Na^+ - HCO_3^-$ symport and Na^+/H^+ antiport exchanger (Fitz, Persico, and Scharschmidt. 1989; Fitz, Lidosky, Weisiger, Xie, Cochran, Grotmol, and Scharschmidt, 1991; Moule and McGivan, 1990). The former could contribute to the V_m by direct effect if the symport were electrogenic, or through a indirect way by increasing the intracellular Na^+ and activating the Na^+/K^+ ATPase if the symport were electroneutral. There is evidence for the existence of a Na^+-HCO_3^- symport in hepatocytes, and its role in determination of hepatocyte basal membrane potential has been confirmed (Fitz, Persico, and Scharschmidt, 1989; Fitz, Lidosky, Weisiger, Xie, Cochran, Grotmol, and Scharschmidt, 1991). As for the Na^+/H^+ exchanger, its activity has been characterized in the basolateral membrane of hepatocytes. This electroneutral exchanger cannot affect membrane potential directly, but may exert an indirect effect via an increase in intracellular Na^+ concentrations leading to activation of the electrogenic Na^+/K^+ ATPase. In rat and mouse liver, glucagon and cAMP stimulate the pump and produce prolonged hyperpolarization. This activation of the Na^+/K^+ pump has been attributed to the primary activation of

the Na^+/H^+ exchanger (Moule and McGivan, 1990). Alternatively, the Na^+/H^+ exchanger might exert its effect on V_m via influencing intracellular pH. Studies have shown that Na^+/H^+ exchange together with a bicarbonate dependent system are important mechanisms for intracellular pH regulation in rat hepatocytes (Henderson, Graf, and Boyer, 1987). In bicarbonate-free medium, the Na^+/H^+ exchanger is responsible for the recovery of the intracellular pH after acid loading (Henderson, Graf, and Boyer, 1987). It has also been shown that hepatocyte V_m is strongly influenced by intracellular pH, with or without changes in extracellular pH. Intracellular acidosis depolarizes and alkalosis hyperpolarizes hepatocyte V_m. These effects of pH on V_m have been suggested to be mediated through changes in plasma membrane K^+ conductance (Fitz, Trouillot, and Scharschmidt, 1989; Henderson, Graf, and Boyer, 1987).

2) Factors that influence hepatocyte V_m:

The V_m of hepatocyte changes in response to a variety of biological stimuli. For instance, changes in V_m have been found to be one of the responses of hepatocytes to certain hormones: both glucagon and epinephrine hyperpolarize hepatocyte V_m (Fitz and Scharschmidt, 1987; Graf and Petersen, 1978), and insulin depolarizes V_m (Wondergem, 1983). An early event in liver regeneration is an ouabain-sensitive hyperpolarization of the membrane

(Wondergem and Harder, 1980.). A higher V_m was found in maternal rat liver than in fetal rats (Chapman and Wondergem, 1984). Hepatocyte V_m also shows difference between sexes: the V_m of male is higher than that of female (Weisiger and Fitz, 1988). Hepatocyte V_m also responds to temperature: within the range of 37 to 27°C, the V_m has been shown to behave as a linear function of temperature and show a depolarization of about 1.6 mV for each degree below 37°C (Wondergem and Castillo, 1986). Hepatocyte V_m varies based on intracellular or extracellular pH: in both cases, acidosis caused depolarization, while alkalosis caused hyperpolarization (Bear and Petersen, 1987; Fitz, Trouillot, and Scharschmidt, 1989). Hepatocyte V_m responds to alanine infusion by an initial depolarization followed by a predominant hyperpolarization (Fitz, and Scharschmidt, 1987). In addition to these, hepatocyte V_m has also been found to change according to the osmolality of the extracellular medium: V_m hyperpolarizes in hypotonic medium and depolarizes in hypertonic medium (Howard and Wondergem, 1987). Although some of the mechanisms of these changes in hepatocyte V_m are not quite clear, it is obvious that hepatocyte V_m are closely related to the cell function.

3) The intracellular mechanisms for changing hepatocyte V_m:

Not all of the mechanisms that different biological factors utilize to induce change in hepatocyte V_m are clear.

The following are some of the mechanisms involved in changing hepatocyte V_m induced by different biological stimuli:

a) Change in membrane K^+ conductance (directly or indirectly): Any stimulus which affects hepatocyte membrane K^+ conductance, G_K, will cause a change in V_m. Examples of it include hypotonic shock- and amino acid uptake induced-hyperpolarization of the hepatocyte V_m (Howard and Wondergem, 1987; Khalbuss and Wondergem, 1990; Kristensen, 1986). There is some evidence suggesting that hepatocytes swell and cell membrane K^+ permeability increases in both of these cases. The intracellular second messenger of this increase in G_K is not clear. It has been proposed that cell membrane stretch or increase in intracellular Ca^{2+} may account for the increased G_K in these cases (Lang, 1989; Bear, 1990). In both liver slices and isolated hepatocytes from guinea-pig, noradrenaline causes a transient hyperpolarization of 5-10 mV which is accompanied by a rapid and transient efflux of K^+ from the cells (Haylett, 1976, Haylett and Jenkinson, 1972). The efflux of K^+ is associated with changes in intracellular Ca^+ concentration. Quinine and apamin, specific inhibitors of Ca^{2+}-dependent K^+ channels in other cell types, block the effect of noradrenaline on K^+ efflux and V_m (Field and Jenkinson, 1987). These findings suggested that α-agonist-mediated increases in intracellular Ca^{2+} concentrations open Ca^{2+}-

dependent K^+ channels in hepatocyte plasma membrane, and thus increasing in K^+ permeability of the membrane and hence the V_m (Moule and McGivan, 1990).

b) Stimulation or inhibition of the Na^+-K^+ ATPase: As has been mentioned previously, the Na^+-K^+ pump can contribute to hepatocyte V_m in two ways. One is directly due to the electrogenic nature of the pump itself. The other is indirectly due to the pump's effect on maintenance of transmembrane K^+ concentration gradient. Generally speaking, any physiological event that may cause an increase in intracellular Na^+ will stimulate Na^+-K^+ pump activity because the pump is sensitive to changes in intracellular Na^+ concentration (Fitz, Lidosky, Weisiger, Xie, Cochran, Grotmol, and Scharschmidt, 1991; Haber, Pressley, Loeb, and Ismail-Beigi, 1987; Van Dyke and Scharschmidt, 1983; Pollack, Tate, and Cook, 1981). The increased pump activity will in turn induce a hyperpolarization of the membrane. For instance, L-alanine uptake in hepatocyte had been suggested to stimulate the activity of the pump due to the influx of Na^+ (Kristensen, 1986). But, the Na^+/K^+ pump also can be activated without any increases in intracellular Na^+. In rat or mouse liver, administration of α-agonists produces a rapid hyperpolarization of V_m accompanied by a transient phase of K^+ uptake up to 5 min duration, followed by a short phase of K^+ release which is complete in a further 5 min. The initial phase of K^+ uptake was associated with Na^+

release and was found to be sensitive to ouabain, indicating that activation of Na^+/K^+ ATPase was involved (Moule, and McGivan, 1990). These results provide good evidence that the hyperpolarization produced by α-agonists is a consequence of an activation of Na^+/K^+ATPase activity. This stimulation of the pump activity was independent of any increases in intracellular Na^+ in rat and mouse liver (Moule, and McGivan, 1990).

c) Stimulation or inhibition of the Na^+/HCO_3^- symport: An electrogenic Na^+/HCO_3^- cotransport mechanism which mediates the influx of Na^+, HCO_3^-, and negative charge under basal conditions has been found in the basolateral membrane of rat hepatocytes (Fitz, Persico, and Scharschmidt, 1989). In cultured rat hepatocyte, an increase in extracellular HCO_3^- at constant Pco_2 causes hyperpolarization; removal of extracellular HCO_3^-, with or without concomitant changes in Pco_2 and extracellular pH, causes a reversible depolarization; removal of extracellular Na^+ causes a depolarization that is inhibited by the presence of SITS or absence of HCO_3^-, is unaffected by amiloride, and persists in the presence of ouabain (Fitz, Persico, and Scharschmidt, 1989). The effects of all these agents or conditions are explained by the electrogenic Na^+/HCO_3^- symport system of hepatocytes.

d) Stimulation or inhibition of the Na^+/H^+ antiport: The Na^+/H^+ antiport is electroneutral and its effect on V_m

is indirect. In perfused rat liver (Friedmann and Dambach, 1980) and isolated segments of mouse liver (Graf and Petersen, 1978), administration of glucagon or cAMP gave rise to a transient membrane hyperpolarization. This hyperpolarization produced by glucagon and cAMP has been suggested to be due to the activation of the Na^+/K^+ pump secondary to the primary activation of the Na^+/H^+ exchanger (Moule and McGivan, 1990). Stimulation of Na^+/H^+ causes an increase in intracellular Na^+ concentration, $[Na^+]_i$. In addition to stimulating the pump, increased $[Na^+]_i$ might induce intracellular alkalosis. This may be another reason for the hyperpolarization of V_m because an increase in V_m has been found to be induced by alkalosis in hepatocyte due to an increased G_K (Fitz, Trouillot, and Scharschmidt, 1989; Henderson, Graf, and Boyer, 1987).

e) Influence of cell Ca^{2+}: Ca^{2+} could be one of the second messengers for K^+ channels in hepatocytes of certain species. In guinea pig and rabbit liver an increase in K^+ permeability via Ca^{2+}-sensitive K^+ channels is observed in response to α-agonists (Moule and McGivan, 1990). Some findings suggested that α-agonist-mediated increases in intracellular Ca^{2+} concentrations open Ca^{2+}-dependent K^+ channels in the hepatocyte plasma membrane, thus increasing K^+ permeability of the membrane and hence the membrane potential (Moule and McGivan, 1990). Certain studies also reported that the presence of Ca^{2+}-dependent K^+ channels in

rat hepatocytes. Using patch-clamp technique, Bear and Petersen (1987) demonstrate that L-alanine evokes opening of single Ca^{2+}-activated K^+ channels in rat liver cells. Bear suggested recently that hypotonic shock or L-alanine uptake stimulates a nonselective cation channel in rat hepatocytes, which leads to the influx of Ca^{2+} into the cell and thus activates the Ca^{2+}-activated K^+ channel (Bear, 1990). Khalbuss and Wondergem reported that both extra- and intracellular Ca^{2+} are involved in control and activation of hypotonic stress-induced increase in mouse hepatocyte V_m (Khalbuss and Wondergem, 1991).

4) The physiological importance of hepatocyte V_m:

Compared with the number of observations of changes in hepatocyte V_m and the mechanisms accounting for these changes, relatively little is known about the physiological significance of hepatocyte V_m. It has been proposed that hepatocyte V_m is important in regulating metabolic processes including gluconeogenesis (Friedmann, Somlyo, and Somlyo, 1971; Friedmann, and Dambach, 1980), amino acid transport (Wondergem and Harder, 1980), and the rate of uptake of bile salts (Edmondson, Miller, and Lumerg, 1985).

Hepatocyte V_m has been demonstrated to be important in energization of amino acid transport in liver (Kristensen, 1980). The uptake of several neutral amino acids is accomplished by Na^+-dependent cotransport systems in

hepatocytes. The Na^+ electrochemical gradient across the cell membrane is critical for these secondary active transport systems and the V_m represents part of this gradient. V_m also plays an important role in hepatocyte uptake of bile acids. A large amount of bile acids are taken up by a multispecific Na^+-dependent bile acid transporter located on the basolateral membrane, and the driving force for this carrier is the Na^+ electrochemical gradient established by Na^+-K^+ ATPase (Nathanson and Boyer, 1991). Boyer (1980) suggested that once the anion accumulates within the cell, the electrochemical gradient tends to drive the anion out of the cell. If the permeability of the canalicular membrane for bile acids were greater than that of the basolateral membrane, bile acids might accumulate preferentially in bile without necessarily requiring an additional input of energy.

Hepatocyte Intracellular K^+ activity (a^i_K)

Intracellular K^+ activity differs from cell K^+ content in that intracellular K^+ activity reflects the effective intracellular K^+ concentration based on its free energy, while the K^+ content deals with the total amount of K^+ within the cell. The freedom of movement of ions is restricted by the immediate ionic environment. The electrostatic attraction between the anions and cations limits the mobility of individual ions and therefore, the

more concentrated a solution, the greater the restriction on ion movement. So, measurement of intracellular ionic activity is more meaningful to biological function than measurement of ion concentration. Moreover, K^+ also may be compartmentalized, either chemically or structurally and, thereby, be immobile. This compartmentalized K^+ contributes to total K^+ content but is not measurable as free ions.

Potassium is a critical component of almost every biological system. All growing cells, either plant or animal, are thought to accumulate potassium (Lubin, 1964). Intracellular K^+ is involved in many interorgan functions. For example, there are well defined patterns of interorgan K^+ movements. Efflux of liver K^+ accompanies peripheral uptake of K^+ in different physiological or pathological conditions, such as hepatic glycogenolysis, reduced hepatic blood flow, stimulation by epinephrine, glucagon, steroids and norepinephrine, acute hypokalemia, blood loss, anoxia, stress, and acute diseases (Shoemaker and Elmyn, 1969). The liver removes K^+ during postprandial absorption, when K^+ is released by the working muscle, during hyperglycemia, after intravenous glucose load, and with stimulation by insulin especially in the presence of hyperglycemia (Shoemaker and Elwyn, 1969).

Intracellular K^+ is vital to mammalian cells. In sarcoma-180 cells, loss of intracellular K^+ has been found to be associated with a parallel depression of the rates of

synthesis of protein and DNA (Lubin, M. 1967). The stimulating effect of potassium on protein synthesis is known to directly influence the level of RNA translation (Horowitz and Lau, 1988). Potassium salt microinjection into Xenopus laevis oocytes mimics gonadotropin treatment on protein synthesis (Lau, Yassin, and Horowitz, 1988). In cultured rat liver slices, certain hepatotoxic agents, which induced decrease in slice K^+ content, also caused a depression in protein synthesis. The level of decrease of intracellular K^+ has been used as an indicator of hepatotoxicity (Smith, Fisher, Shubat, Gandolfi, Krumdieck, and Brendel, 1987).

Potassium is also involved in cell volume regulation. A regulatory volume decrease in hypotonic media associated with a substantial net loss of KCl has been reported in Ehrlich ascites cells (Hendil, and Hoffmann, 1974; Hoffmann, 1978). The K^+ loss observed during regulatory volume decrease has been demonstrated to reflect a volume-induced increase in apparent K^+ permeability of the cell membrane in different cell types (Hoffmann, Simonsen, and Lambert, 1984). In hepatocytes, amino acids uptake induced increase in K^+ permeability and net loss of intracellular K^+ content has been demonstrated (Kristensen, 1986). This increased K^+ permeability has been suggested to be responsible for the alanine uptake-induced hyperpolarization of the V_m in hepatocytes and the increased efflux of intracellular K^+

(Kristensen, 1986). It has been proposed that these comprise part of the cell's response to osmotic imbalance and regulate volume. Thus, it is clear that a better understanding of hepatic a^i_K in response to different extracellular stimuli is necessary for comprehension of liver metabolic functions.

Hepatocyte Intracellular Cl^- activity (a^i_{Cl})

In many epithelia, the steady state intracellular Cl^- activity (a^i_{Cl}) has been found to exceed the equilibrium level predicted by the Nernst equation. This implies the presence of active Cl^- transport mechanisms in the cell membrane. Active transport of Cl^- has been described in a variety of epithelia including the gallbladder, trachea, small intestine, and kidney (Fitz, and Scharschmidt, 1987). Two of the well characterized active transporters for Cl^- are: 1) the electroneutral Na^+-Cl^- or Na^+-K^+-$2Cl^-$ cotransporter and 2) the electrically silent Cl^-/HCO_3^- exchanger. Activating either of these transporters generates an inward flux of Cl^-.

Unlike the tissues mentioned above, efforts in finding the active transporter for Cl^- on the basolateral membrane of hepatocyte failed in getting a positive result. On the other hand, most studies have shown that the measured intracellular Cl^- activity is close to the Cl^- equilibrium potential predicted by the Nernst equation (Lyall, Croxton,

and Armstrong, 1987; Fitz, and Scharschmidt, 1987; Graf, et al. 1987). These findings favor the passive distribution of Cl^- with the V_m in hepatocytes. Recently, the passive distribution nature of hepatocyte Cl^- has been further confirmed by current clamp technique in hepatocyte couplets (Graf, Henderson, Krumpholz, and Boyer, 1987).

In hepatocytes, the membrane permeability for Cl^- is very high. Cl^- was found to cross the liver cell membrane rapidly (Moule, Bradfold, and McGivan, 1987). Some studies suggested a conductivity order of $G_{Cl} > G_K > G_{Na}$ in mouse liver slices (Graf and Petersen, 1978). Lyall et al. (1987) observed rapid depletion of intracellular Cl^- in mouse hepatocytes by substituting extracellular Cl^- with gluconate. Their data indicate that the hepatocyte membrane possesses a high conductance to Cl^- that allows intracellular Cl^- to equilibrate with that of the external medium according to the Nernst equation (Lyall, Croxton and Armstrong, 1987). The high Cl^- permeability of the plasma membrane provides the physical basis for the passive distribution of Cl^- with membrane potential.

The physiological role of hepatocyte intracellular Cl^- is unclear yet. In addition to being the major anion in the bile, recent studies have indicated that Cl^- plays a critical role in the mechanism of regulatory volume decrease in rat hepatocytes (Haddad, Beck, Boyer, and Graf, 1991; Corasanti, Gleeson, and Boyer, 1990). Hepatocyte

intracellular Cl^- was also found to decrease during alanine uptake (Fitz, and Scharschmidt, 1987).

One of the major functions of liver is bile secretion. The formation of canalicular bile by the hepatocyte is believed to result from active solute transport followed by the passive flow of water (Scharschmidt, Van Dyke, and Steves, 1982). Cl^- is the predominant anion in hepatic bile and is present in concentrations of 90-100 mM (Fitz and Scharchmidt, 1987). According to the available data, hepatocyte intracellular Cl^- activity is in the range of 15 to 35 mM (Fitz. and Scharschmidt, 1987; Lyall, Croxton, and Armstrong, 1987). Although there has been a report suggesting the existence of Cl^-/HCO_3^- on the canalicular membrane (Meier, Knickelbein, Moseley, Dobbins, and Boyer, 1985), little is known about hepatic Cl^- transport. More work needs to be done for a full understanding of Cl^- transport and its role in the intracellular metabolism in liver.

Theory of Special Methods Used in This Study

Electrophysiological technique is used in this study. Double-barreled microelectrodes are used throughout the experiments. One barrel works as the conventional microelectrode for measuring V_m and the other barrel is used as the ion-selective microelectrode for measuring intracellular ion activities. After filling these barrels

with certain solutions, the Ag/AgCl wires to which a high input impedance (>10^{14} ohms) preamplifier was connected were then inserted in the micropipette. These micropipettes were then placed into the hepatocytes via a micromanipulator. The micropipettes when filled with KCl actually function as salt-bridges across cell membrane, connecting a Ag/AgCl half-cell to the intracellular compartment. The voltage of this half-cell is measured relative to a reference Ag/AgCl half-cell in the tissue bath. In a Ag/AgCl electrode, the reversible reaction is:

$$Ag + Cl^- \longleftrightarrow AgCl + e^-$$

The function of the silver chloride is to provide a stock of chloride ions in solid form, ready for use should the reaction tend to proceed to the left. This keeps a charge from building up on the surface and thus prevents the half-cell potential from drifting during the experiment.

Another important aspect of measuring transmembrane potential is the electrical resistance of the tip. A series of constant current pulses of 0.5 nanoampers was injected through the open-tip microelectrode during my measurements in order to calculate the tip resistance. By measuring the voltage drop across the tip during the current pulse, the resistance could be calculated by Ohms Law (Resistance = Voltage / Amperes). The electrodes were selected with tip

resistances around 10 megohms. It was found that at this resistance there was less clogging of the tip during the impalement, which resulted in a stable measurement. It is also possible to calculate the approximate tip diameter by measuring the resistance of the tip. The smaller the diameter of the tip, the greater the electrical resistance. The double-barreled microelectrode tip diameters in my experiments had a mean value of 1.25 ± 0.07 μm measured by scanning electron microscopy.

Some of the uncertainties in trying to measure the correct V_m are problems with junction potential and tip potentials. The junction potential is the potential produced across the end of a broken microelectrode, across the junction between the solution inside the pipette and the solution outside the electrode. Tip potential may be defined as the potential difference observed between microelectrode and reference (macro) electrode filled with the same solution when both are immersed in the same medium. In terms of simple electrodiffusion theory, the tip potential should be negligible. However, it is frequently quite large (30-40 mV) and may abruptly change during the course of an experiment. The tip potential is thought to originate from charge effects of the wall of the glass micropipette. Tip potentials are frequently a source of problems because, due to the different ionic environments inside versus outside the cell, it is not known whether or

how much the tip potential will change when a cell is impaled. Therefore, a basic criterion for acceptable impalements is an unchanged tip potential when the microelectrode tip is returned from the cell interior to the external medium. Another criterion for the acceptability of recordings is sharp deflection of the microelectrode tracing upon penetration of the cell. With these criteria, it is assumed that real magnitude of V_m could be approximated closely from repeated impalements in a given preparation.

Ion-selective microelectrodes have the advantage of being able to measure specific ion activity in a direct, fast, and continuous way. Other advantages include excluding organelles because of the small tip size, and conducting measurements in vivo. There are, however, disadvantage and difficulties. The preparation of the electrode is not as simple as that of the conventional microelectrode. It takes experience and skill to achieve success in making these electrodes. In making these ion-selective microelectrodes, one barrel is first made hydrophobic by N,N-dimethyltrimethylsilylamine vapor. This is important for two reasons. If the barrel is not coated well and, thus is not hydrophobic, water can displace the ion-exchanger from the tip. Another problem is that an aqueous layer could exist between the exchanger and the micropipette wall. This could provide a low resistance pathway which would attenuate the voltage across the ion-

exchanger.

Generally speaking, the liquid ion-exchanger is produced by dissolving a specific certain ionic carrier, which normally contains both hydrophobic and hydrophilic conformational characteristics, in an organic solution. Ion-selective electrodes are selective because the liquid ion-exchanger membrane that separates the two half-cells will conduct only when it is in the presence of that specific ion. Also, they do not measure the ion concentration but measure the activity of that particular ion. Because electrostatic attraction between the anions and the cations restrict their freedom of motion. The mobility of the ions is restricted, and this restriction is greater, the greater the total concentration of ions in the solution. Only ions interact with the ion exchanger in the tip of the electrode resulting in a potential difference. But certain ion-selective microelectrodes are subject to interference from other ions. This problem will be discussed later.

Another problem in these experiments is the electrical interference due to the very high resistance of the microelectrodes. Extensive shielding is required along with good earth grounding in order to prevent 60 Hertz line energy and static electricity from interfering with the recordings. Because of the high resistance of the microelectrodes, there is also a longer time constant for

the resistance-capacitance (RC) network at the tip. This delays the information from the cell to the recorder. Since the time course of the V_m and intracellular ionic activity changes are on the order of seconds or minutes, they are longer than the time constant delay of the electrodes, this problem is negligible. The potential difference measured with the ion-selective microelectrode is the sum of the V_m and the voltage due to the difference between intracellular and extracellular ionic activities. In order to measure ion activities, the double-barreled microelectrode was used to record the V_m and ion activity voltage simultaneously. These two voltages were then run through a differential amplifier to obtain the difference in potential in order to calculate the ion activity of the cell. The use of double-barreled microelectrodes adds to the series of uncertainties when measuring ion activity due to the different sizes of the tips and thus different tip potentials. Certain criteria have been set up in these experiments which will be explained in more detail in a later chapter.

CHAPTER 2

GENERAL METHODS

Materials and Animals

Adult, male mice (ICR strain) were purchased from Charles River Breeding Labs. (Charles River, MA) and were fasted 12-18 h before experiments. The vivarium housing the mice met all standards of the American Association for Accreditation of Laboratory Animal Care. All inorganic chemicals were purchased from Fisher Scientific (Pittsburgh, PA), and organic chemicals were purchased from either Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO).

Liver slice preparation, maintenance and temperature control

Mice were killed by cervical dislocation and the left-lateral or median lobe of the liver was removed quickly and placed on gauze which was moistened with 0.9% NaCl. A glass microscope slide was pressed gently onto the lobe to keep it from moving while a slice of 1 mm thick was made by using a razor blade clamped in a hemostat. A slice of 5 * 5 mm surface area was placed into an acrylic tissue chamber and held down by a small, steel ring washer placed onto the slice. This ensured that microelectrode impalements, taken within the ring, were consistently of cells on the encapsulated, uncut surface of the slice.

Liver slices for control V_m measurements were

superfused with Krebs physiological solution. Temperature at the slice was monitored continuously with a thermistor positioned next to the tissue, and it was maintained at 37°C by passing solution at 12-15 ml/min through tubing coiled within a temperature-controlled water jacket that formed the base of the tissue chamber. A solenoid valve controlled switching between control and experimental solutions. A diagram of the tissue slice chamber, microelectrodes, and electrical recording equipment is shown in Fig. 1.

Solutions Used in the Experiments and Osmolality Control

Krebs physiological solution contained (in mM) 103 NaCl, 4.7 KCl, 2.56 $CaCl_2$, 1.3 $MgCl_2$, 25 $NaHCO_3$, 1.15 NaH_2PO_4, 2.8 glucose, 4.9 sodium pyruvate, 4.9 sodium glutamate, 2.7 sodium fumarate, and was equilibrated with 95% O_2-5% CO_2 (pH 7.46). Hyperosmotic conditions were created by adding 100 mM sucrose to normal Krebs physiological solution.

For those experiments where hyposmotic condition was used, modified Krebs physiological solution contained (in mM) 52.5 NaCl, 4.7 KCl, 2.56 $CaCl_2$, 1.3 $MgCl_2$, 25 $NaHCO_3$, 1.15 NaH_2PO_4, 2.8 glucose, 4.9 sodium pyruvate, 4.9 sodium glutamate, 2.7 sodium fumarate, 103 sucrose, and was equilibrated with 95% O_2-5% CO_2 (pH 7.46). Hyposmotic condition was created by reducing 50 or 80 mM sucrose from Krebs physiological solution.

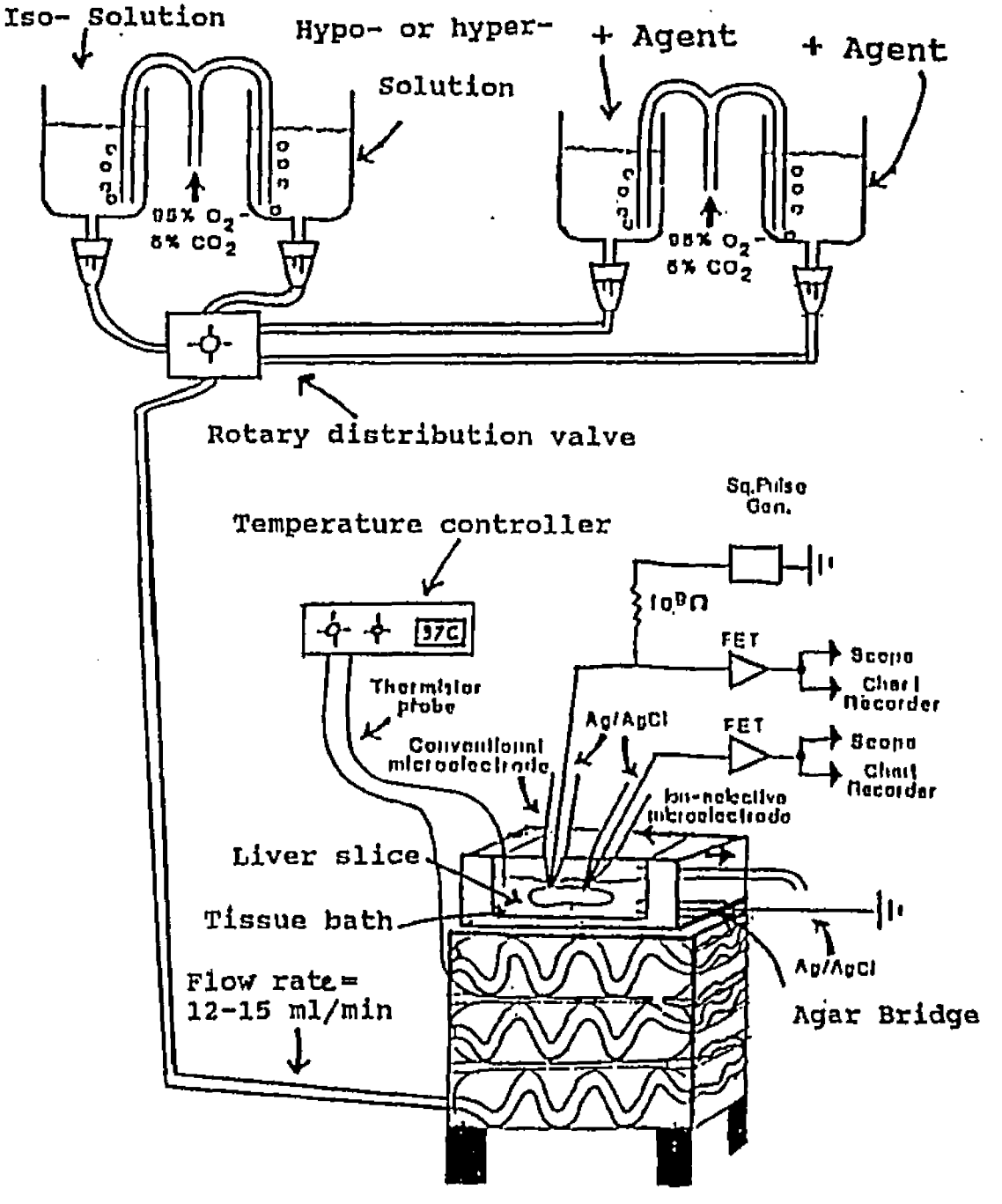

Fig. 1. Schematic diagram of experimental procedure.

For those experiment where the effect of L-alanine was tested, 20 mM of L-alanine was added to the normal Krebs physiological solution, and the osmolality in the control solution was adjusted by adding 20 mM sucrose.

The osmolality of each experimental and control solution was measured with an automatic osmometer based on the principle of freezing point depression (Precision Systems, Natick, MA.).

Solution Used for Loading Hepatocytes with TMA^+

For those experiments where tetramethylammonium (TMA^+) ion was measured, liver slices were loaded with TMA^+ by perfusing the tissue with Krebs solution where 100 mM sucrose was replaced by 50 mM TMA^+-chloride for 10 to 15 min. In order to maintain the intracellular TMA^+ concentration constant, 5 mM NaCl was replaced by 5 mM TMA^+ in both control and experimental Krebs solutions.

Fabrication of Double-Barrelled Ion Sensitive Microelectrodes

Double-barrelled ion-selective microelectrodes were prepared from fiber-filled, borosilicate double-capillaries (1.2 mm o.d., 0.6 mm i.d., A-M systems, Everett, WA). They were cleaned first by boiling for 15 min in 500 ml of distilled water plus tree drops of liquid detergent (Liquinox). After a 1-h tap water rinse, the microfilament

capillaries were boiled for 15 min in distilled water and then dried at 90°C in a forced-air oven. The double-barrelled microelectrodes were pulled in a vertical pipette puller (700D, D. Kopf, Instr., Tujunga, CA) and had tip-diameters of approximately 1.25 ± 0.07 μm (n=6) measured by scanning electron microscopy. Ion-selective microelectrodes were prepared as follows: one barrel from the blunt end of the double-barrelled micropipette was ground 1 mm shorter than the other. The longer end was inverted and stuck into a patent, tapered hole in the lid of a teflon jar. A few drops of N,N-dimethyltrimethylsilylamine (Fluka, Ronkonkoma, NY) were put into the jar, which then was covered with the lid holding the micropipettes. The jar was placed into a forced-air oven at 25°C for 24 min, allowing the compound in the jar to evaporate and coat the inside of each barrel inserted into the lid. Then the micropipettes were put into a holder and dried in the oven at 100°C for 12 hr. Liquid ion-exchanger, Corning 477317 for K^+ and TMA^+, and Corning 477913 for Cl^-, was introduced into the back of the silylated micropipette, filling the microelectrode tip with a column of exchanger ranging from 0.2-1.0 mm. The microelectrodes were back-filled with 0.5 M KCl. The open-tip (reference) barrel was filled with 1 M Na formate.

For those double-barrelled Na^+ selective microelectrodes, Na^+ exchanger was introduced into the silylated micropipette the same way as it was for K^+- and

Cl^--selective microelectrodes. Then, instead of KCl, 0.5 M NaCl was used to fill the rest of Na^+ sensitive barrel. The open-tip (reference) barrel was filled with 0.5 M KCl. The Na^+ exchanger was made by dissolving 10% (by volume) of the Na^+-selective ligand [N,N',N"-Triheptyl-N,N',N"-trimethyl-4,4',4"-propylidynetris (3-oxabutyramide)] in 3-nitro-o-xylene containing about 1 percent of tetrachlorophenylborate. The later one is a large hydrophobic anion used to improve the response time of the exchanger (O'Doherty, Garcia-Diaz, and Armstrong, 1979).

All microelectrodes were connected by Ag-AgCl half-cells to a high input impedance ($>10^{14}$ MΩ) preamplifier with unit gain (515L Analogue Devices, Norwood, Ma). Reference electrode (bath) consisted of a Ag-AgCl half-cell connected to the chamber by an agar (4% in Krebs medium) bridge. Voltage were recorded by a digital voltmeter (Keithly Instr., Cleveland, OH), a storage oscilloscope (Tektronix, Beaverton, OR), and a strop-chart recorder (Western Graphtec, Irvine, CA).

Calibration of K^+-Sensitive Microelectrodes and Intracellular K^+ Activity Measurements

K^+-selective microelectrodes were calibrated in 100, 50, 20, 10, 5 and 1 mM KCl solutions. One of the representative calibration curves for K^+-selective microelectrode is shown in Fig. 2. For electrode

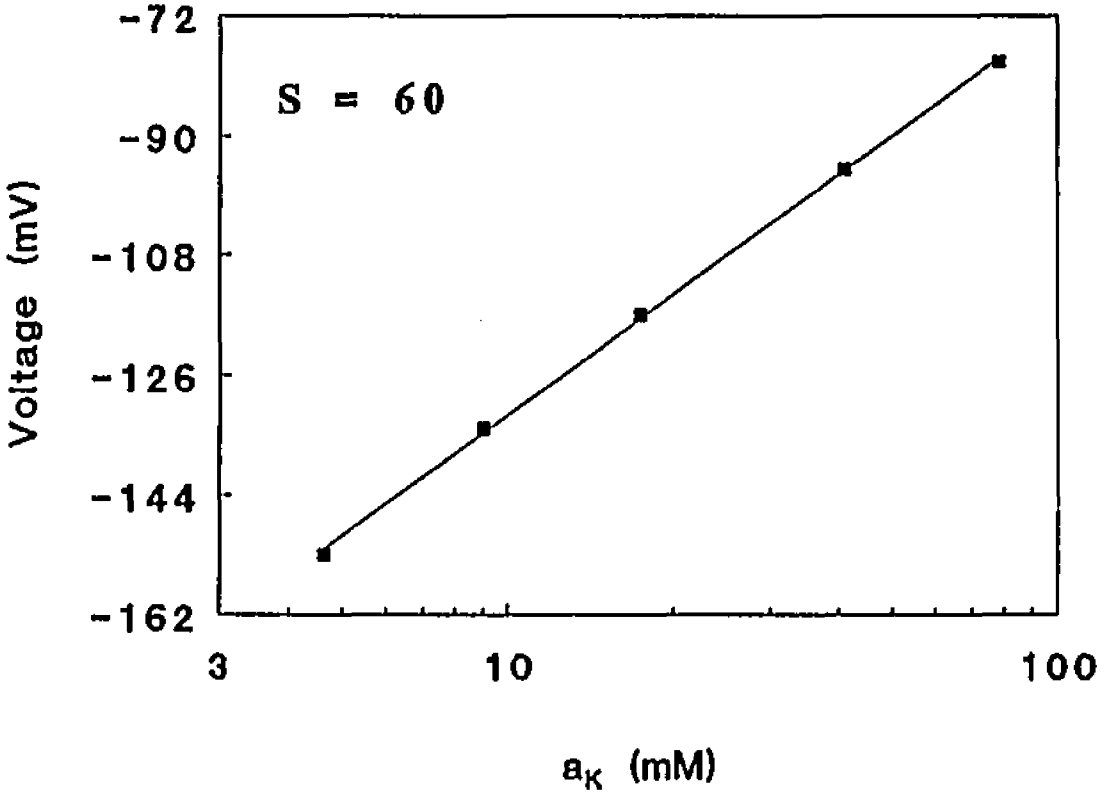

Fig. 2. A representative calibration curve for double-barreled K^+-selective microelectrode calibrated in 5, 10, 20, 50, and 100 mM pure KCl solutions. S = the slope of the calibration curve.

calibrations, the slopes of voltage vs K^+ activity ranged from 54 to 61 mV/10-fold change in K^+ activity, with a mean ± S.E. of 58.0 ± 0.5 (n=15). Microelectrode selectivities for K^+ over Na^+, ranging from 29 to 70 with a mean ± S.E. of 43.0 ± 4.5 (n=8) were determined by:

$$k_{KNa} = 10^{(VK-VNa)/S} \quad (1)$$

where K_{KNa} is the selectivity coefficient of the microelectrode for K^+ over Na^+, S is the slope of the microelectrode calibration in pure KCl and V_K and V_{Na} are the microelectrode voltages in pure, 100 mM solutions of the respective ions.

The regression of the K-selective microelectrode potential, V_K, vs log a_K in pure KCl is:

$$V_K = S*\log a_K + b \quad (2)$$

Thus, a^i_K is computed by direct interpolation solving for:

$$a^i_K = 10^{(VK-Vm-b)/S} \quad (3)$$

where V_K and V_m are potentials recorded in the intracellular space with K-selective microelectrodes and open-tip microelectrodes, respectively. S and b are slope and intercept of the regression line.

When measuring the K^+ activities with K^+-selective microelectrodes, in most cases at least three impalements were made in each liver slice, which was obtained consistently from a different animal. In each impalement, membrane potential and V_K were measured continuously and were recorded before, during and after the osmotic stress. Anisosmotic conditions were imposed onto the liver slices only after the membrane potentials and the V_K became stable. The same applied when switching back to control solution from the anisosmotic situations. In a few instances, either to reduce the risk of breaking the microelectrode tips or when experiencing difficulty finding a hepatocyte within a liver slice with a satisfactory V_m, microelectrodes were maintained in the same cell for a subsequent measurement after switching back to control solution from the anisosmotic solution.

Calibration of Cl^--Sensitive Microelectrodes and Intracellular Cl^- Activity Measurement

Cl^--selective microelectrodes were calibrated in KCl solutions covering the physiologic or intracellular range. Cl^- activities in these calibration solutions were computed by modifications of the Debye-Hückel equation (Croxton and Armstrong, 1992; Lyall, Croxton, and Armstrong, 1987). One of the representative calibration curves for Cl^--selective microelectrode is shown in Fig. 3.

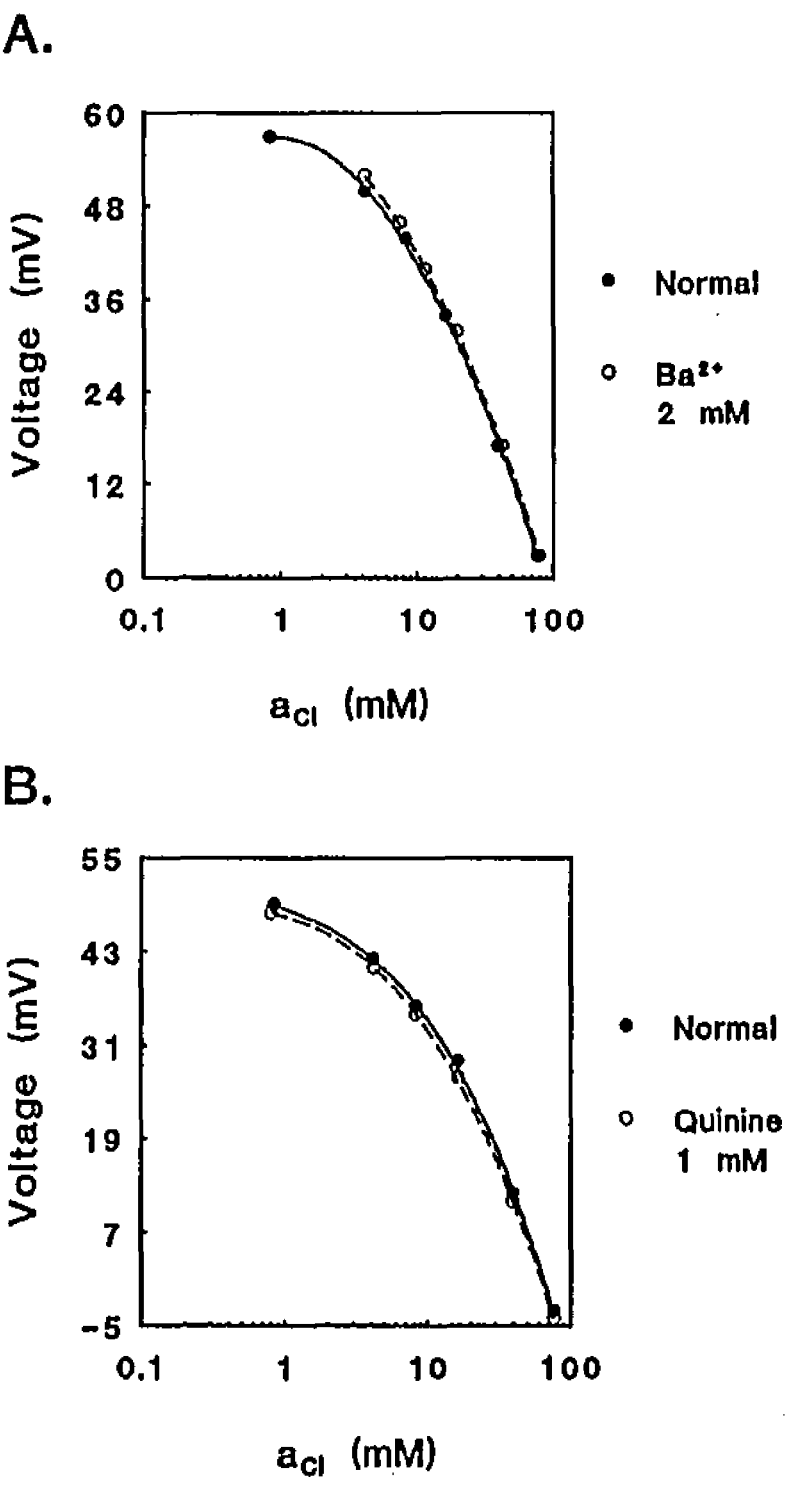

Fig. 3. Representative calibration curves for double-barreled Cl^--selective microelectrodes. A. Filled circles show calibration in 1, 5, 10, 20, 50, and 100 mM KCl solutions each contains 25 mM $NaHCO_3$, 4.9 mM Na pyruvate, 4.9 mM Na glutamate, and 2.7 mM Na fumarate. Open circles show same plus 2 mM $BaCl_2$ added to each mixture. B. Filled circles show same as in A. Open circles show same plus 1 mM quinine added to each mixture.

Cl^--selective microelectrodes are subject to interference by extracellular and intracellular anions, notably HCO_3^- and bile salts, respectively (Lyall, Croxton, and Armstrong, 1987). For this reason, Cl^--microelectrodes were calibrated in 100, 50, 20, 10, 5, and 1 mM KCl solutions each containing fixed amounts of 25 mM $NaHCO_3$, 4.9 mM Na pyruvate, 4.9 mM Na glutamate, and 2.7 mM Na fumarate. The latter are the possible interfering anions of the extracellular Krebs medium.

The Cl^--sensitive microelectrodes were calibrated according to the methods described by Lyall, Croxton, and Armstrong (1987). Calibration curves were fitted to the Nicolsky-Eisenman equation according to Croxton and Armstrong (1992):

$$E = E^0 + (RT/z_iF)\ln(a_i + \sum K_{ij}^{Pot} a_j^{(z_i/z_j)}) \qquad (4)$$

where E is the potential of the ion-selective electrode, E^0 is a reference potential, a_j is the activity of ions type j and K_{ij}^{Pot} is a potentiometric selectivity coefficient of the electrode for type j ions compared with type i. Other variables have their usual meanings. Making the substitutions:

$$S = (RT/z_iF)\ln(10) \qquad (5)$$

$$DL = \sum K_{ij}^{pot} a_j^{(z_i/z_j)} \quad (6)$$

we obtain:

$$E = E^0 + S \log (a_{Cl} + DL) \quad (7)$$

Thus,

$$a^i_{Cl} = 10^{(VCl - Vm - Vo)/S} + DL \quad (8)$$

where V_{Cl} is the intracellular potential of the Cl^- electrode, Vm is the cell membrane potential, V_o is the reference potential of the chloride electrode in the bath, $S = (RT/z_iF)\ln 10$, and DL is a detection limit whose theoretical value equals the sum of the products of selectivity coefficients of interfering ions compared with Cl^- times the activity of the interfering ions. DL is estimated by a least-squares fit using a Newton-Raphson algorithm of Cl^- selective microelectrode calibration curve obtained in the presence of interfering anions. Thus, DL estimates the total interfering ions in the calibrating solutions and sets a limit to the accuracy with which intracellular Cl^- can be measured (Croxton and Armstrong, 1992; Lyall, Croxton, and Armstrong, 1987). The mean value of DL for 33 Cl^--selective microelectrodes in my experiments is 4.2 ± 0.6 mM. As a result, my estimates of a^i_{Cl} are

slight underestimates if this degree of interference does not occur intracellularly.

When measuring the Cl^- activities with Cl^--selective microelectrodes, usually three impalements were made in each liver slice obtained consistently from a different animal. In each impalement, membrane potential, and V_{Cl} were measured continuously and steady-state values were recorded before (control) and during (experimental) the osmotic stress. The experimental values were obtained 4 to 5 min after osmotic stress and at least one min after stable voltages had been reached. Anisosmotic conditions were imposed onto the liver slices only after the control V_m and the V_{Cl} became stable. In a few instances, either to reduce the risk of breaking the microelectrode tips or when having trouble finding a hepatocyte within a liver slice with a satisfactory V_m, microelectrodes were maintained in the same cell for a later measurement after switching back to control solution from the anisosmotic solution.

Calibration of Na^+-Sensitive Microelectrodes and Intracellular Na^+ Activity Measurement

Na^+-selective microelectrodes were subject to interference from extracellular Ca^{2+} and from intracellular K^+, where a^i_K is ~10-fold greater than a^i_{Na} (Wondergem, and Castillo, 1986). Consequently, a^i_{Na} was determined by interpolating intracellular voltages of the Na^+-selective

microelectrodes onto calibration curves obtained from electrode voltages in varying NaCl solutions (2-100 mM), each containing a fixed amount of KCl at 132 mM. Activity of the later (100 mM) approximates the measured a^i_K by Wondergem and Castillo (1986). Slopes of linear portions of calibration plots from 3 Na^+-selective microelectrodes ranged from 56 to 66 mV/10-fold change in Na^+ activity, with a mean ± SE of 62 ± 3 mV/10-fold change in Na^+ activity. One of the representative calibration curves for Na^+-selective microelectrode is shown in Fig. 4. Microelectrode selectivities for Na^+ over K^+, computed from these slopes by the fixed-interference method (Armstrong, and Garcia-Diaz, 1980; Saunders and Brown, 1977), ranged from 54.9 to 77.5 with a mean ± SE = 62.4 ± 7.5 (n = 3).

Calibration of TMA^+-selective microelectrode

The calibrations of TMA^+-selective microelectrode were carried out at room temperature in TMA^+ solutions whose concentrations covered the expected range of a^i_{TMA} for hepatocytes loaded with TMA^+. These solutions comprised 1, 5, 10, 20 and 50 mM TMA chloride, plus 125 mM KCl in each solution. TMA^+ activities in these calibration solutions were computed according to the Debye-Hückel equation as modified by Armstrong et al. (Armstrong, Byrd, and Hamang, 1973) and according to data of Conway (Conway, 1969). The

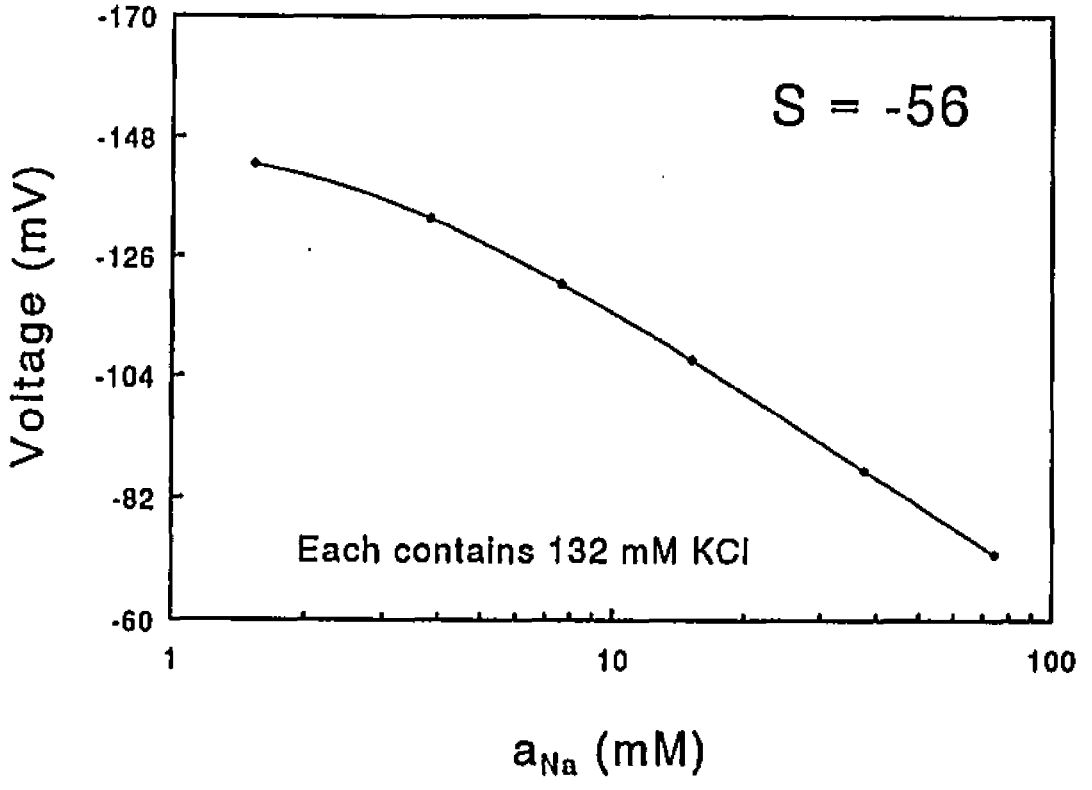

Fig. 4. A representative calibration curve for double-barreled Na^+-selective microelectrode calibrated in 5, 10, 20, 50, and 100 mM NaCl solutions each contains 132 mM KCl. S = the slope of the calibration curve.

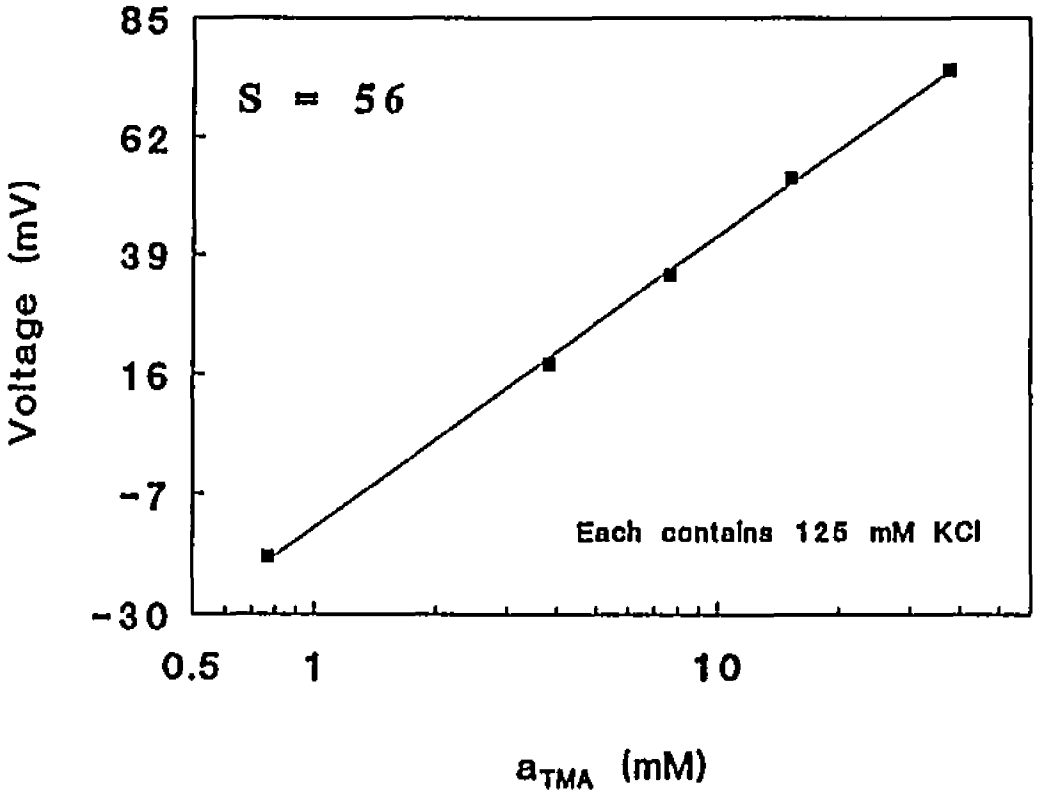

Fig. 5. A representative calibration curve for double-barreled TMA^+-selective microelectrode calibrated in 1, 5, 10, 20, and 50 mM TMA^+ chloride solutions each contains 125 mM KCl. S = the slope of the calibration curve.

average slope of electrode voltage versus TMA^+ for all experiments was 61 ± 1 (S.E., n = 6). One of the representative calibration curves for TMA^+-selective microelectrode is shown in Fig. 5. Intracellular TMA^+ activity was computed by direct interpolation solving for:

$$a^i_{TMA} = 10^{(VTMA-Vo-Vm)/S} \quad (9)$$

where a^i_{TMA}=intracellular TMA^+ activity, V_{TMA}=intracellular voltage of the TMA^+-selective microelectrode. V_0=voltage at the intercept of the calibration curve, V_m=transmembrane potential, S=slope of the calibration curve.

Criteria for Effective Loading Hepatocytes with TMA^+

To measure the change of cell volume with this TMA^+ loading technique, certain criteria must be met: (1) loading hepatocytes with TMA^+ must not permanently alter the plasma membrane's relative ion permeabilities; (2) the a^i_{TMA} in hepatocytes loaded with TMA^+ must remain constant during the intracellular recording period. The second point had been met practically by maintaining 5 mM TMA^+ in the perfusate (Adorante, and Miller, 1990).

Determination of Changes in Hepatocyte Intracellular Water Volume

Change in cell water volume based on TMA^+ measurements

is computed by Cotton and co-workers (Cotton, Weinstein, and Reuss, 1989) to be:

$$\Delta V = V_t - V_0 = V_0[(a^i_{TMA})_o/(a^i_{TMA})_t - 1] \quad (10)$$

where ΔV_t=change in cell water volume at time t, V_0=initial cell water volume, $(a^i_{TMA})_o$=intracellular TMA^+ activity at time zero, $(a^i_{TMA})_t$=intracellular TMA^+ activity at time t. Nonetheless, mouse hepatocytes have a large range of initial volumes due to their polyploid nuclei, which range from 2n to 16n (Carriere, 1969). Consequently, I did not assign an initial cell volume to serve as a reference for all measurements and comparisons. Instead, I chose to compare the ratios of $(a^i_{TMA})_o/(a^i_{TMA})_t$ measured under control conditions with ratios obtained under experimental conditions, where $(a^i_{TMA})_o$=intracellular TMA^+ activity at time zero, and $(a^i_{TMA})_t$=intracellular TMA^+ activity at time when the V_m became stable after onset of osmotic stress.

Criteria for Valid Impalements and V_m Measurements with Conventional and Double Barreled Microelectrodes

Criteria for valid impalements included: (1) rapid deflection of the voltage trace on advancing the microelectrode into the cell; (2) an intracellular voltage recording that was stable within 2 mV; (3) return of the voltage trace to within 2 mV of baseline when the

microelectrode was withdrawn; and (4) resting V_m for the control > 20 mV. Constant current pulses (0.5 nA; 300 ms duration) were passed periodically through the open-tip microelectrode to assess electrode resistance during the course of an intracellular measurement. Irreversible increases in microelectrode resistance indicated clogging of the microelectrode and results were disregarded. Hepatocytes show extensive low-resistance intercellular communication, presumably through gap junctions. Thus, input resistance measurements comprise membrane resistance and intercellular resistance. Also, the cellular component of input resistance in mouse liver slices is often 10^2 less than microelectrode resistance. These facts disallowed inference about changes in membrane resistance from changes in input resistance.

At least three impalements were made for each individual animal (each liver slice). In each impalement, V_m was measured continuously and was recorded before and during the osmotic stress, respectively. Hyper- and hyposmotic conditions were imposed onto the liver slices only after the membrane potentials became stable. The same applied when switching back to control solution from the anisosmotic solutions. Microelectrodes were repositioned after each impalement to ensure that consecutive measurements were not in the same cell.

Statistical Analysis of the Data

Multiple comparisons of means were accomplished by Least Significant Difference procedure (Sokal and Rohlf, 1969), and difference between two means is accomplished by paired comparisons of student t-test (Sokal and Rohlf, 1969). Relative changes of intracellular ion activities were represented by the experimental/control values. Ratios of steady-state values were chosen because previous work demonstrated substantial variation of hepatocyte V_m and ionic activities between animals (Wondergem, R., and L. B. Castillo, 1986). Paired measurements were made in the same liver slices. Results are expressed as mean ± S.E. Linear regression analysis was achieved by the least-squares computation and the significance of the regression coefficients was determined by the t-test at n-2 degrees of freedom and at $P<0.05$.

CHAPTER 3

MOUSE HEPATOCYTE MEMBRANE POTENTIAL AND INTRACELLULAR CHLORIDE ACTIVITY DURING OSMOTIC STRESS

INTRODUCTION

Isolated rat hepatocytes respond to hypotonic swelling by a regulatory volume decrease (RVD) during which they actively shrink back to or toward their original volume (Bakker-Grunwald, 1983; Corasanti, Gleeson, and Boyer, 1990). Mechanisms of this regulatory decrease in cell volume comprise loss of cell KCl and osmotically obligated water (Corasanti, Gleeson, and Boyer, 1990; Haddad and Graf, 1989).

Hepatocyte volume regulation in the mouse also involves changes in transmembrane potential, V_m, (Howard and Wondergem, 1987; Khalbuss and Wondergem, 1990). Variations in plasma membrane K^+ conductance, g_K, account for osmotic stress-induced changes in hepatocyte V_m (Wang and Wondergem, 1991). For example, the hyperpolarization of V_m during hyposmotic stress is inhibited by Ba^{2+} and quinine, which are known K^+ channel blockers (Howard and Wondergem, 1987; Khalbuss and Wondergem, 1990). These g_K-dependent variations in V_m are essential to hepatocyte volume regulation (Khalbuss and Wondergem, 1990).

Notwithstanding the importance of cation fluxes and V_m

changes for hepatocyte volume control, very little is known about the role of intracellular anions in the mechanism of volume regulation in hepatocytes. Macroscopic electroneutrality must be preserved during cation fluxes so that equal amounts of anion cross the cell membrane (Grinstein, Clarke, Dupre, and Rothstein, 1982). Corasanti et al (1990) recently evaluated a critical role for intracellular Cl^- in the RVD of rat hepatocytes. They showed that following exposure to hypotonic medium, isolated rat hepatocytes swelled as osmometers within 30-60 s and subsequently underwent RVD toward control volume. Either high extracellular K^+ concentration, barium, or quinine blocked the recovery. Cl^- depletion inhibited RVD by 40% while 0.5 mM DIDS blocked the recovery by almost 90%. Their findings suggest that quinine- and Ba^{2+}-sensitive K^+ conductance and DIDS-sensitive anion conductance, which is partly accounted for by Cl^-, mediate RVD in rat hepatocytes (Corasanti, Gleeson, and Boyer, 1990). A recent report from Haddad et al (1991) showed that a step decrease in external Cl^- accelerates the initial rate of RVD more than twofold; whereas, depleting the cells of Cl^- abolished RVD. Although there is a disagreement between these studies on the relative inhibition of RVD in Cl^--depleted hepatocytes, their results have confirmed a role for Cl^- in the hepatocyte RVD mechanism.

Cl^- distributes passively in liver (Fitz, and

Scharschmidt, 1987; Graf, Henderson, Krumphole, and Boyer, 1987; Lyall, Croxton, and Armstrong, 1987). Thus, in accord with Cl^- permeability of hepatocyte membrane being high (Moule and McGivan, 1990), Cl^- is in steady-state electrochemical equilibrium with V_m. As a result, a more negative intracellular environment will facilitate the Cl^- exit. Mouse hepatocyte V_m exhibits a sustained hyperpolarization with hypotonic shock (Howard and Wondergem, 1987) and during prolonged alanine uptake (Wondergem and Castillo, 1988). So, I proposed that hyperpolarization of V_m provides electromotive force for efflux of Cl^-, which in turn contributes osmotically to RVD in hepatocytes. To test this, I have measured V_m and intracellular Cl^- activities, a^i_{Cl}, by electrophysiological methods during osmotic stress in mouse liver slices. My results show hepatocyte a^i_{Cl} is in electrochemical equilibrium with V_m during both hyper- and hyposmotic stresses. I conclude that redistribution of a^i_{Cl} by changes in V_m contribute to hepatocyte volume regulation in the mouse.

RESULTS

Hepatocyte V_m in Isosmotic Media and During Osmotic Stresses

Hepatocyte V_m was -39 ± 0.7 mV, (n = 16 animals) in normal Krebs physiological solution containing 103 mM NaCl (Table 1). In the medium where 50.5 mM NaCl was replaced by 103 mM sucrose (containing 52.5 mM NaCl), the V_m was -28 ± 1.0 mV, (n = 13 animals) (Table 1).

Osmotic stresses to hepatocytes, induced by changing the concentration of sucrose in the medium without altering its ionic strength, elicited changes in V_m consistently. Hepatocyte V_m depolarizes during hypertonic stress. A hyperosmotic stress of 1.4 times that of control decreased hepatocyte V_m by 18 mV from -39 ± 1 to -21 ± 1 mV (n = 16 animals) (Fig. 6A; Table 1). On the other hand, hepatocyte V_m hyperpolarizes during hypotonic stress. A hyposmotic stress of 0.7 times that of control hyperpolarized hepatocyte V_m by 64% from -28 ± 1 to -46 ± 1 mV (n = 13 animals) (Table 1; Fig. 6B).

The Effects of Osmotic Stresses on a^i_{Cl}

In order to test my hypothesis, that hepatocyte intracellular Cl^- distributes passively with the change of V_m, intracellular Cl^- activities were measured the same time as the change of V_m with double-barreled microelectrodes. The results are shown in Table 1. Hyperosmotic stress

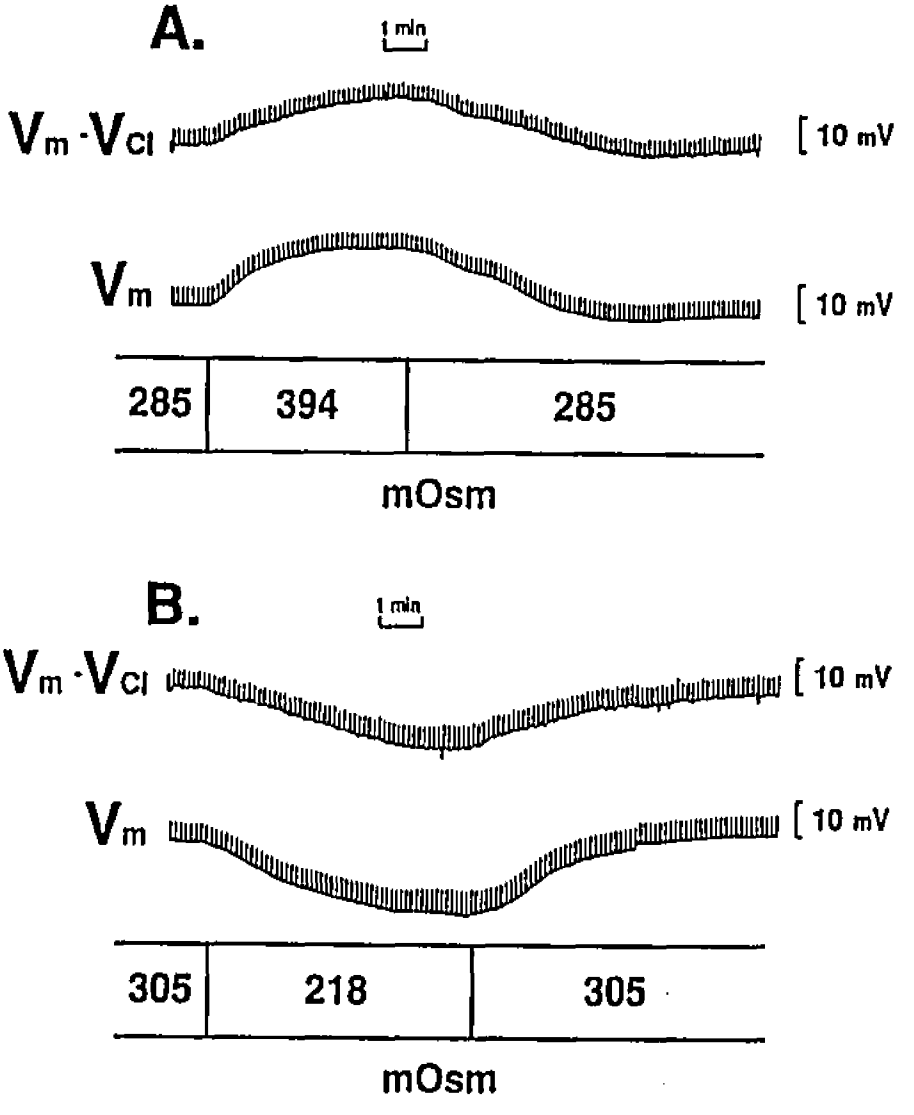

Fig. 6. Representative intracellular recordings of time-dependent changes in hepatocyte V_m and V_m-V_{Cl} in response to A. hyperosmotic stress by added sucrose or B. hyposmotic stress by deleting sucrose. Intermittent deflections in the voltage traces result from 0.5 nA of current passed through the reference (V_m) electrode to assess microelectrode resistance. Increased electronegativity is downward in all traces. V_m-V_{Cl} = -(V_{Cl}-V_m), thus upwards shows increasing a^i_{Cl} and downward shows decreasing a^i_{Cl}. Time-base applies to both traces.

Table 1. Effect of osmotic stress on steady-state intracellular Cl^- activity and transmembrane potential

	V_m (-mV)	a^i_{Cl} (mM)	E_{Cl} (-mV)	π_e/π_e
Control	39±1	19±2	38±0.3	1.4
Hyperosm.	21±1*	38±3*	19±2*	(n=16)
Control	28±1	17±1	26±2	0.71
Hyposm.	46±1*	8±1*	46±3*	(n=13)

*Differs from control value at $p < 0.001$; n = No. of animals. Values of V_m and a^i_{Cl} were obtained 4-5 min after switching to hypo- or hyperosmotic conditions, and after voltages had stabilized for at least 1 min.

causes an increase in hepatocyte intracellular Cl^- activity, a^i_{Cl}. A hypertonic shock of 1.4 times that of control induced a 2 fold increase in a^i_{Cl}, from 19 ± 2 to 38 ± 3 mM (n = 16; Table 1) in my experiments. A representative trace for the hyperosmotic stress induced change in intracellular potentials, V_m and $V_m - V_{Cl}$, is shown in Fig. 6.A. Changes with time of $V_m - V_{Cl}$, which were proportional to changes in a^i_{Cl}, nearly paralleled changes in V_m.

Hyposmotic stress decreases hepatocyte intracellular Cl^- activity, a^i_{Cl}. A hypotonic shock of 0.71 times that of control induced a 0.52 fold decrease in a^i_{Cl}, from 17 ± 1 to 8 ± 1 mM (n = 13; Table 1) in my experiments. A representative trace for the hyposmotic stress induced change in intracellular potentials, V_m and $V_m - V_{Cl}$, is shown in Fig. 6B. Again, changes with time of $V_m - V_{Cl}$, which were proportional to changes in a^i_{Cl}, nearly paralleled changes in V_m.

These observations are consistent with passive distribution of a^i_{Cl} due to osmotic stress-induced changes in hepatocyte V_m.

Hepatocyte Chloride Equilibrium during Osmotic Stress

The Cl^- equilibrium potential (E_{Cl}) decreased with hyperosmotic stress (1.4 times control Osm) from -38 ± 0.3 mV to -19 ± 2 mV (n = 16 animals; Table 1); whereas, hyposmotic stress increased E_{Cl} from -26 ± 2 mV to -46 ± 3 mV

(n = 13 animals; Table 1). These values for E_{Cl} agreed well with the corresponding control, hyperosmotic, and hyposmotic values for V_m. The differences between E_{Cl} and V_m for each group were well within experimental error set by the 4.2 mM detection limit for Cl-sensitive microelectrodes.

Passive distribution of hepatocyte a^i_{Cl} with V_m during osmotic stress is demonstrated further by plotting log a^i_{Cl} as a function of V_m. This is shown for control and hyperosmotic conditions in Fig. 7, and for control and hyposmotic conditions in Fig. 8. The Nerstian slope (zF/RTln10) of -0.0164 fell within the 95% confidence limits of the computed regression coefficients for data shown in both Figs. 7 and 8. Taken together all measurements and computations support the hypothesis that hepatocyte Cl^- distributes passively across the plasma membrane, in accord with V_m.

The Effect of Barium on Osmotic Stress-Induced Changes of V_m and on Osmotic Stress-Induced Changes of a^i_{Cl}

To test further the passive distribute of Cl^- with V_m, V_m and a^i_{Cl} were measured when the osmotic stress-induced changes in V_m were blocked by 2 mM Ba^{2+}. Barium inhibits the osmotic stresses induced changes of V_m by blocking the K^+ channel in hepatocytes. In paired comparisons, a 39% decrease in V_m, from -36 ± 1 to -22 ± 1 mV, was induced by hyperosmotic stress [1.4 times control Osm (280 mOsm)].

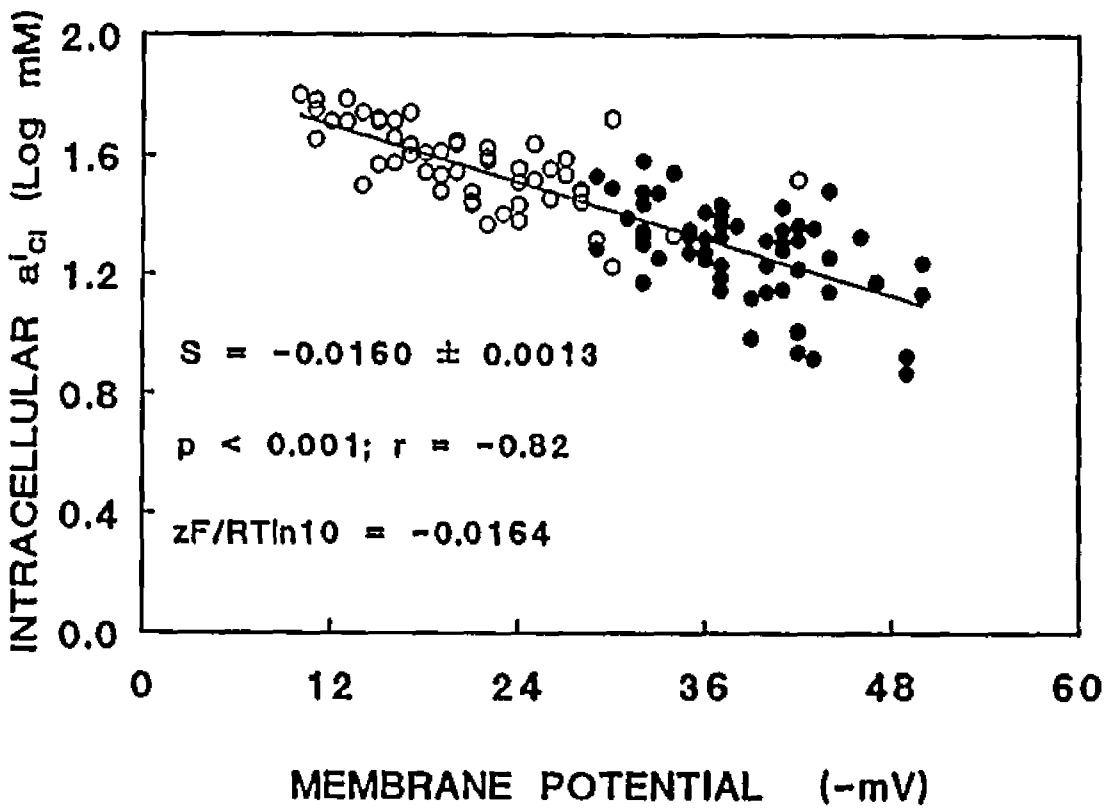

Fig. 7. Regression of hepatocyte a^{i}_{Cl} on V_m in isosmotic control medium (●) and hyperosmotic medium (○) 1.4 times that of control. Solid line shows the best fit for all data points by linear regression analysis.

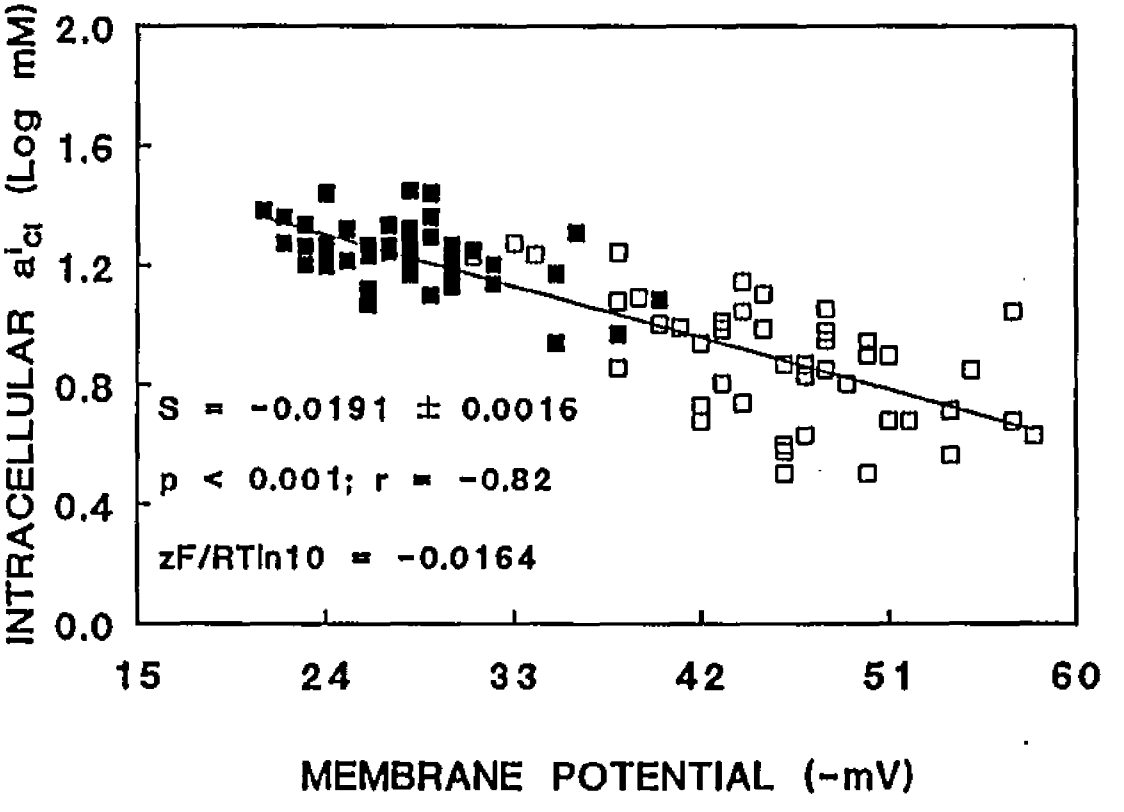

Fig. 8. Regression of hepatocyte a^{i}_{Cl} on V_m in isosmotic control medium (■) and hyposmotic medium (□) 0.71 times that of control. Solid line shows the best fit for all data points by linear regression analysis.

With 2 mM barium present in the solution, the same hypertonic shock caused a 41% depolarization of V_m, from -34 ± 2 to -20 ± 1 mV. During hyposmotic stress [0.71 times control Osm (290 mOsm)], V_m increased 60%, from -30 ± 4 to -48 ± 3 mV in control group. With 2 mM barium present during hypotonic stress, V_m increased by only 20%, from -25 ± 2 to -30 ± 4 mV (Table 2).

In the same paired experiments as mentioned above, hepatocyte intracellular Cl^- activity increased by 67%, from 18 ± 2 to 30 ± 4 mM during hyperosmotic stress. With the presence of 2 mM barium, the same hyperosmotic stress caused a similar increase in a^i_{Cl}, from 18 ± 2 to 31 ± 2 mM. On the other hand, during hypotonic stress, hepatocyte intracellular Cl^- decreased by 53%, from 15 ± 3 to 7 ± 1 mM in control group. In the presence of 2 mM barium, the same hypotonic stress induced only a 26% decrease in a^i_{Cl}, from 19 ± 2 to 14 ± 2 mM. One of the representative traces showing the inhibitory effects of Ba^{2+} is shown by Fig. 9.

Again, these observations are consistent with passive distribution of a^i_{Cl} due to osmotic stress-induced changes in hepatocyte V_m.

The Effect of Barium Chloride on Hyposmotic Stress Induced Changes of V_m and a^i_{TMA}

To distinguish the inhibitory effect of Ba^{2+} on the hypotonic stress induced change of a^i_{Cl} from that of cell

Table 2. Effect of Quinine (0.5 mM) & Barium (2 mM) on the Changes of V_m, E_{Cl}, & a^i_{Cl} Induced by Osmotic Stresses

Condition	V_m (-mV)	E_{Cl} (-mV)	a^i_{Cl} (mM)	π_e / π_e
Isosmotic	36 ± 1	38 ± 4	18 ± 2	1
Hyperosmotic	22 ± 1[5]	25 ± 3	30 ± 4[2]	1.4
Isosmotic + Barium	34 ± 2	38 ± 3	18 ± 2	1
Hyperosmotic + Barium	20 ± 1[5]	23 ± 2	31 ± 2[2]	1.4
Isosmotic	30 ± 4	31 ± 6	15 ± 3	1
Hyposmotic	48 ± 3[1]	51 ± 6	7 ± 1[1]	0.7
Isosmotic + Barium	25 ± 2	23 ± 3	19 ± 2	1
Hyposmotic + Barium	30 ± 4	32 ± 4	14 ± 2	0.7
Isosmotic	37 ± 3	38 ± 6	18 ± 4	1
Hyperosmotic	20 ± 3[3]	17 ± 3	41 ± 5[1]	1.4
Isosmotic + Quinine	30 ± 2	33 ± 4	22 ± 3	1
Hyperosmotic + Quinine	21 ± 3	19 ± 5	38 ± 7	1.4
Isosmotic	29 ± 1	29 ± 3	15 ± 2	1
Hyposmotic	51 ± 3[4]	55 ± 3	6 ± 1[4]	0.7
Isosmotic + Quinine	27 ± 1	30 ± 3	14 ± 2	1
Hyposmotic + Quinine	29 ± 2	36 ± 1	11 ± 0	0.7

Differs from control value at: [1]$p < 0.05$; [2]$p < 0.025$; [3]$p < 0.01$; [4]$p < 0.005$; [5]$p < 0.001$. Each value is the mean ± SEM for three animals.

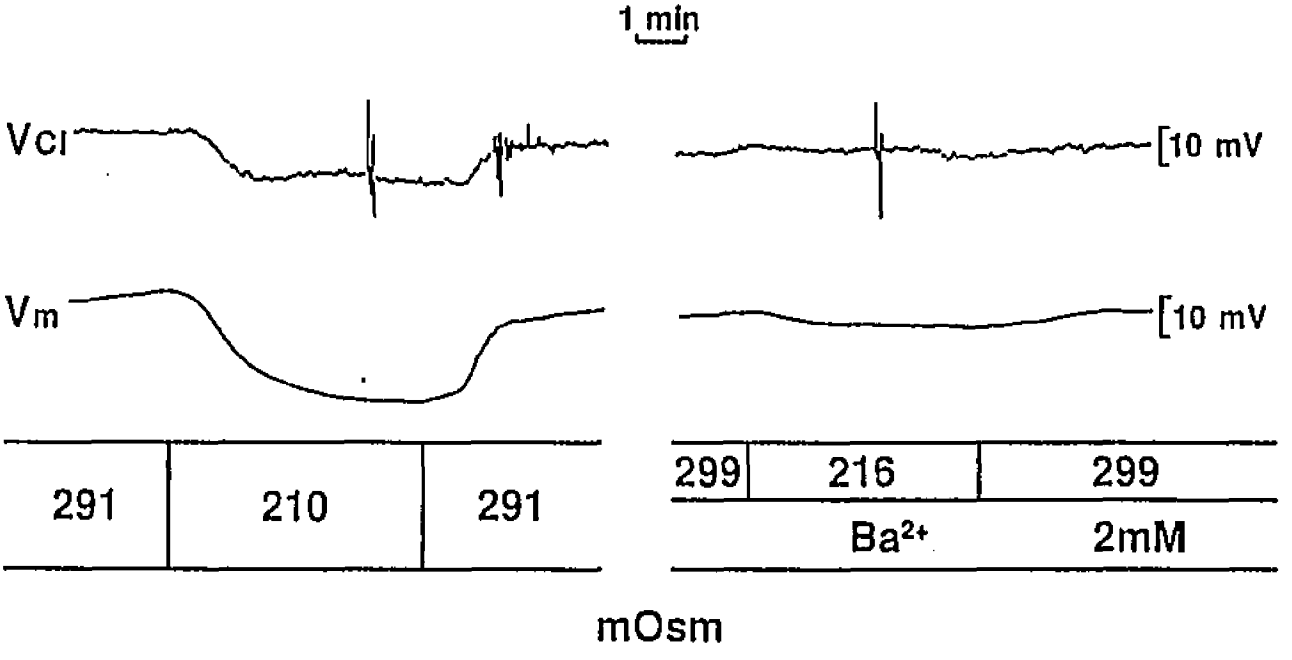

Fig. 9. Representative intracellular recordings of V_m and V_{Cl} during hyposmotic stress without and with $BaCl_2$ (2 mM). Greater electronegativity is downward in all traces. Time scale applies to all traces.

water volume change caused by Ba^{2+} itself during hyposmotic stress, a^i_{TMA} was measured in both control and Ba^{2+} present conditions during hypotonic stress. The results were shown in Table 3. In these paired experiments, hyposmotic stress of 0.82 times control osmolality induced a 70% hyperpolarization, from -26 ± 3 to -45 ± 5 mV and a 9% decrease in a^i_{TMA}, indicating a 10% increase in hepatocyte intracellular water volume (Table 3, Fig. 10A). In the presence of 2 mM Ba^{2+}, the same hyposmotic stress only induced a 25% increase in V_m, from 20 ± 1 to 26 ± 1 mV. Meanwhile the a^i_{TMA} decreased 10% indicating a 11% increase in intracellular water volume (Table 3, Fig. 10B). Thus, it is unlikely that effects of Ba^{2+} on a^i_{Cl} results from changes in cell volume in response to hypotonic stress.

The Effect of Quinine on Osmotic Stress Induced Changes of V_m and a^i_{Cl}

Based on the same principle as mentioned above, a^i_{Cl} was measured when osmotic stress-induced changes of V_m were inhibited by 0.5 mM quinine. In paired comparisons, a 46% decrease in V_m, from -37 ± 3 to -20 ± 3 mV, was induced by hyperosmotic stress [1.4 times control Osm (280 mOsm)]. With 0.5 mM quinine present in the solution, the same hypertonic shock caused a 30% depolarization of V_m, from -30 ± 2 to -20 ± 3 mV. During hyposmotic stress [0.71 times control Osm (290 mOsm)], V_m increased 76%, from -29 ± 1 to

Table 3. Effect of Ba^{2+} (2 mM) on hypotonic stress induced changes of V_m and a^i_{TMA} (n=3)

	V^c_m (-mV)	V^e_m (-mV)	V^e_m/V^c_m	$V^e_m-V^c_m$ (-mV)	a^c_T (mM)	a^e_T (mM)	a^e_T/a^c_T	a^e_T/a^c_T-1 (mM)	π^e/π^c
Untreated	26±3	45±5*	1.70±0.02	18±2	14±3	13±3‡	0.91±0.02	0.10±0.02	0.82
Ba^{2+}	20±1	26±1[d*]	1.25±0.05	5±1	22±7	20±6	0.90±0.01	0.11±0.01	0.82

V^c_m=hepatocyte transmembrane potential. V^e_m=transmembrane potential in hyposmotic experimental solution. a^c_T=hepatocyte intracellular TMA^+ activity in isosmotic control solution. a^e_T=intracellular TMA^+ activity in hyposmotic experimental solution. a^e_T/a^c_T-1 represents the intracellular water volume change induced by hyposmotic stress. All values are averages ± S.E.; values for individual experiments are from at least three separate measurements, whereas the mean values are averages of the values from each animal. ‡ Differs from control, P<0.01. * Differs from control, P<0.001. [d*] Differs from untreated value, P<0.001.

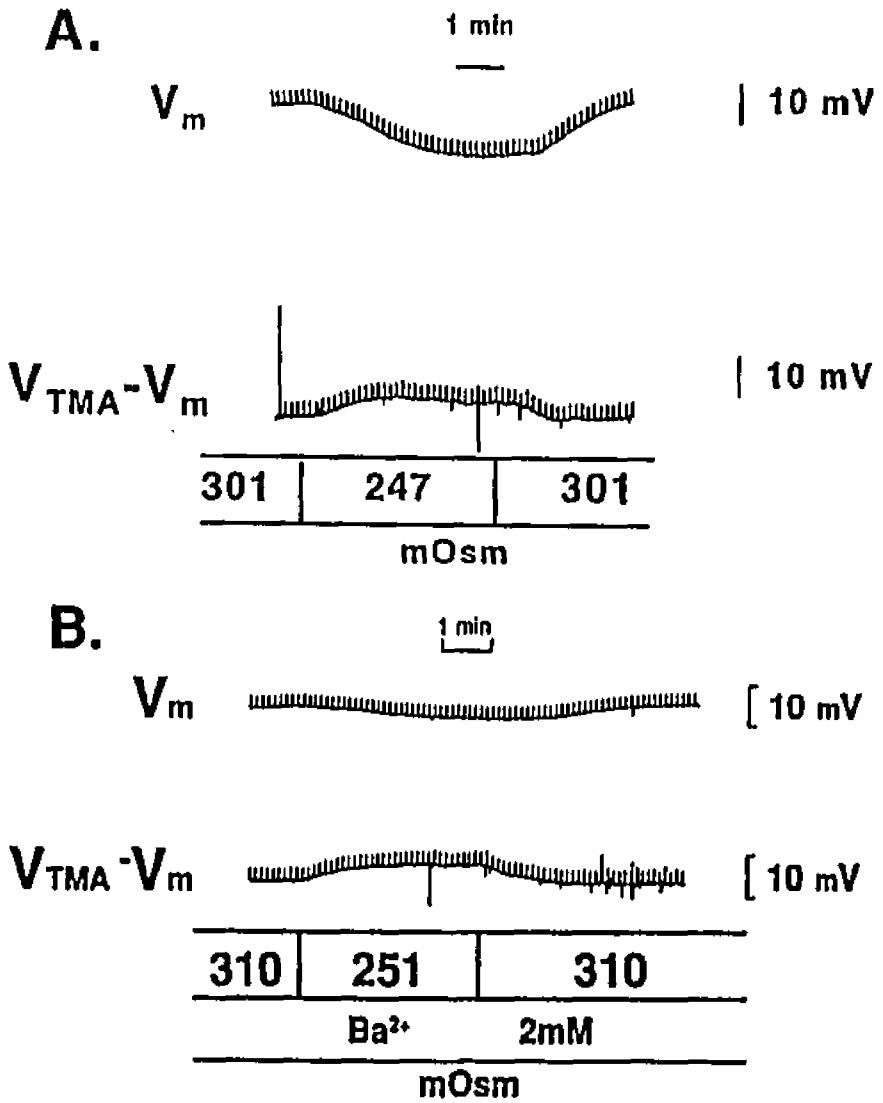

Fig. 10. Representative intracellular recordings of time-dependent changes in hepatocyte V_m and V_{TMA}-V_m during hyposmotic stress without and with $BaCl_2$ (2 mM). Greater electronegativity is downward in all traces. The upward deflections of V_{TMA}-V_m in both A. and B. indicate the decrease of a^i_{TMA}.

-51 ± 3 mV in control group. With 0.5 mM quinine present during hypotonic stress, V_m increased by only 7%, from -27 ± 1 to -29 ± 2 mV (Table 2).

In the same paired experiments, hepatocyte intracellular Cl^- activity increased by 128%, from 18 ± 4 to 41 ± 5 mM during hyperosmotic stress. With the presence of 0.5 mM quinine, the same hyperosmotic stress caused a 73% increase in a^i_{Cl}, from 22 ± 3 to 38 ± 7 mM. During hypotonic stress, hepatocyte intracellular Cl^- decreased by 60%, from 15 ± 2 to 6 ± 1 mM in control group. When 0.5 mM quinine was present in the solution, the same hypotonic stress only induced a 21% decrease in a^i_{Cl}, from 14 ± 2 to 11 ± 0 mM.

DISCUSSION

Hepatocyte V_m Depolarizes During Hyperosmotic Stress and Hepatocyte V_m Hyperpolarizes During Hyposmotic Stress

Howard and Wondergem (1988) have demonstrated that alteration of extracellular NaCl concentration induces changes of hepatocyte V_m. Increasing NaCl concentration of the medium depolarizes hepatocyte V_m; whereas, reducing medium NaCl concentration hyperpolarizes V_m. These changes in V_m have been attributed to the alteration of the osmolalities of the media. However, these experiments did not distinguish the effects on hepatocyte V_m of altering media osmolality from those induced by changing in media ionic strength. Here, I was able to test the effects of pure osmotic stresses on hepatocyte V_m and on intracellular ion activities by changing sucrose concentration of the media. The design was based on the impermeability of hepatocyte plasma membrane to sucrose (Alpini, Garrick, Jones, Nunes, and Tavaloni, 1986). My results demonstrated an inverse relationship of hepatocyte V_m and medium osmolality by showing that hepatocyte V_m depolarizes during hyperosmotic stress and hyperpolarizes during hyposmotic stress. As it will be discussed later, these changes in V_m are at least in part due to the changes of G_K of the cell membrane.

The behavior of hepatocyte V_m during osmotic stresses

is not what I predicted based on changes in ionic equilibrium calculated by the Nernst equation. The most predictable explanation for osmotic stress-induced change in V_m is a passive phenomenon due to change in the transmembrane ionic electrochemical equilibrium, particularly that for K^+ (Wang and Wondergem, 1991). Since hepatocytes shrink and a^i_K increases during hypertonic shock, I expected that the increased a^i_K would induce a hyperpolarization of V_m due to the increased K^+ electrochemical gradient across the cell membrane. Instead, I observed that hepatocyte V_m depolarized with hyperosmotic stress (Wang and Wondergem, 1991). Based on the same principle I would expected hepatocyte V_m to depolarize during hypotonic shock. Instead, I observed a hyperpolarization of the V_m, which is quite different from the depolarization of V_m that accompanies hyposmotic stress in Erlich ascites tumor cells (Lang et al., 1987). The uniqueness of the V_m response to the osmotic stress in hepatocytes suggests regulatory mechanisms in addition to change in K^+ equilibrium potential. For example, hyperosmotic stress-induced hepatocyte shrinkage might inhibit or close the stretch-activated K^+ channel and this would cause a depolarization of the membrane. Hepatocyte membrane K^+ conductance have been shown to decrease during hyperosmotic stress (Wang and Wondergem, 1991). Hyposmotic stress, on the other hand, causes hepatocyte swelling. This

would activate stretch-activated K^+ channels and induce a hyperpolarization of the membrane. The hypotonic shock induced increase in hepatocyte membrane K^+ permeability has been previously suggested by different laboratories (Howard and Wondergem, 1987; Khalbuss and Wondergem, 1990; Cohen and Lechene, 1990; Berthon, Claret, and Mazet, 1980). Although stretch-activated K^+ channels have not been reported in hepatocyte membrane, volume-regulatory K^+ movements have been characterized in liver perfused with hypotonic or hypertonic media, and it seems likely that such ion fluxes occur via stretch-sensitive K^+ channels (Lang, et al., 1989).

A possible physiological significance of my findings is that the changes of V_m alone could contribute to the volume regulatory responses of hepatocytes to different osmotic stresses. For instance, the changes in hepatocyte V_m might provide an electromotive force for the redistribution of Cl^- during osmotic stresses since Cl^- is passively distributed with V_m in hepatocytes. Thus, hypertonic shock induced depolarization of V_m should cause an increase in the intracellular Cl^- concentration, while hypotonic shock induced hyperpolarization of V_m should lead to a decrease in intracellular Cl^- concentration. In both cases, the directions of Cl^- fluxes will favor the recovery of the hepatocyte volume from those changed by osmotic stresses. Another possible function of osmotic stress-induced changes

in V_m could include regulating certain voltage-sensitive ion channels in the membrane and, thereby, regulate intracellular metabolic processes. Changes in hepatocyte V_m could alter both the getting of membrane Ca^{2+} channels and the driving force for Ca^{2+} influx.

Barium Inhibits the Changes of V_m Induced by Osmotic Stresses

As it has been mentioned before, Ba^{2+} has been demonstrated to be effective as a typical K^+ channel blocker in many tissues including hepatocytes (Latorre and Miller, 1983; Graf, Haddad, Haussinger, and Lang, 1988). Ba^{2+} blocks the K^+ channel with exceptionally high affinity. The potential blocking effect of Ba^{2+} on K^+ channel has been suggested to be due to the fact that Ba^{2+} has a very similar unhydrated crystal diameter to that of K^+ (0.270 for Ba^{2+} and 0.267 nm for K^+, respectively). In the channel, Ba^{2+} can interact with the same sites as K^+ does. But since Ba^{2+} has a higher positive charge than does K^+, it binds more tightly to the selective filter itself (Latorre, and Miller, 1983).

Several studies have shown that the changes of V_m during osmotic stresses are due to changes of hepatocyte membrane K^+ conductance (Berthon, Claret, and Mazet, 1980; Kristensen, 1986; Howard and Wondergem, 1987; Cohen and Lechene, 1990; Wang and Wondergem, 1991). In my

experiments, the blocking effect of Ba^{2+} on the changes of V_m during osmotic stresses is consistent with the findings of others and further confirms the involvement of K^+ channels in the osmotic stress-induced changes of V_m in mouse hepatocytes.

Quinine Blocks the Changes of V_m Induced by Osmotic Stresses

Quinine is known to block the Ca^{2+}-dependent K^+ channels in many tissues. Quinine blocks the K^+ efflux from guinea-pig hepatocytes induced by ATP, noradrenaline, and A23187 without affecting the changes in ^{45}Ca movement. It was concluded that quinine is able to block either the Ca^{2+}-dependent K^+ channels present in guinea-pig and rabbit liver cell membranes or the mechanism that controls them (Burgess, Claret, and Jenkinson, 1981). In rat hepatocytes, studies have shown that quinine inhibits L-alanine-induced K^+ efflux. Since the efforts to obtain direct support for the presence of Ca^{2+}-activated K^+ channels in rat hepatocytes is unresolved (Kristensen and Folke, 1984), the existence of a "quinine-sensitive K^+ pathway" has been suggested (Kristensen and Folke, 1984). In mouse hepatocytes, quinine has been found to inhibit the L-alanine-induced repolarization-hyperpolarization of V_m and amino acid transport (Wondergem and Castillo, 1988).

Quinine's effects on K^+ transport are complicated. In human red cells, ^3H-quinine appears to be taken up

nonspecifically by the cells, perhaps in the bilayer, with no evidence of saturation up to 1 mM and no apparent KCl-sensitive component of binding. The proposed mechanism assumes that quinine exerts its action by competitively displacing K^+ from an external binding site, the reported K^+-activation site for the Ca^{2+}-mediated K^+-permeability of the membrane (Reichstein and Rothstein, 1981). Smith and Levinson (1989) have shown that quinine inhibits multiple Na^+ and K^+ transport mechanisms in Ehrlich ascites tumor cells. In their experiment, quinine also leads to membrane depolarization and inhibition of Na^+/K^+ active transport, Na^+-K^+-$2Cl^-$ cotransport and Na^+/H^+ antiport. It is not clear whether these effects involve direct interaction of quinine with the transport systems or whether secondary coupling is involved. The evidence from their investigation argues strongly against the sole use of quinine for evaluating the role of Ca^{2+}-dependent K^+ channels (Smith and Levinson. 1989). In my experiment, the osmotic stress-induced V_m changes are inhibited by quinine, and this is consistent with the findings that quinine inhibits K^+ flux reported by others. There are two interesting things that need to be pointed out in my work. One is that the inhibitory effects of quinine on the osmotic stress-induced changes of V_m seem to be different between hyper- and hyposmotic stress. The percent inhibition in hyposmotic stress is much higher than that of hyperosmotic stress. The other is that the

inhibitory effects of quinine on hepatocyte resting membrane potential also seem to be different in these two conditions. The inhibitory effects of quinine on resting V_m is higher in the hyperosmotic control condition than in the hyposmotic control condition (Table 2). Since V_m decreases to -20 mV in both control and experimental groups during hyperosmotic stress, it is likely that the "inhibitory effect" of quinine is due to its inhibitory effect on the resting V_m in experimental group. As mentioned previously, the NaCl concentrations in these experiments are different. Higher NaCl was used in the hyperosmotic experiment than that of the hyposmotic experiment. So it is likely that quinine's inhibitory effects on resting V_m and on the osmotic induced changes of V_m is dependent on the external NaCl concentration. Interestingly, Reichstein and Rothstein also reported, in human red blood cells, that the inhibitory potency of quinine on K^+ efflux was found to depend on the external K^+ and internal Na^+ concentration of the cell (Reichstein and Rothstein, 1981).

Barium Blocks the Changes of a^i_{Cl} Induced by Osmotic Stresses

The data from my experiments clearly show that Ba^{2+} inhibits the hyperpolarization of V_m induced by hyposmotic stress. Meanwhile, the change of intracellular Cl^- activity is also blocked in this case (Table 2, Fig. 9). As mentioned previously, Ba^{2+} is a well characterized K^+

channel blocker. It is most likely that the inhibitory effect of Ba^{2+} on V_m changes in my experiments is a direct consequence of its blocking effect on the K^+ channels on the cell membrane. I conclude from my results that K^+ permeability of hepatocyte membrane increases during the condition of hypotonic shock. However, the inhibitory effect of Ba^{2+} on the changes of a^i_{Cl} in these experiments most likely is an indirect effect of Ba^{2+}, due to its blocking effect on the changes of V_m. This conclusion is supported by the facts that: 1) Cl^- is passively distributed with V_m in hepatocyte. 2) Ba^{2+}'s effect on hepatocyte intracellular water volume change cannot explain its effect on a^i_{Cl} change during hypotonic shock. 3) RVD in isolated perfused rat liver is associated with a large Ba^{2+}-sensitive efflux of K^+ and a concomitant release of Cl^- (Haddad, Beck, Boyer, and Graf, 1991).

The reason for the failure of Ba^{2+} in blocking the changes of V_m and a^i_{Cl} during hyperosmotic stress in my experiment (Table 2) is unclear. One possibility is that an electrogenic pathway other than the Ba^{2+}-sensitive K^+ channel might play a central role in determination of V_m during hyperosmotic stress. Another possibility may depend on the way in which Ba^{2+} blocks the K^+ channels. For instance, Ba^{2+} might exert its effect on those closed K^+ channels preventing them from opening. On the other side, Ba^{2+} might have less effect on those already opened K^+

channels.

The Effect of Barium on Intracellular Water Volume Change During Hyposmotic Stress

To distinguish the inhibitory effects of Ba^{2+} on the changes of intracellular a^{i}_{Cl} induced by hypotonic stress from that on the cell volume change caused by Ba^{2+} treatment itself, hyposmotic stress-induced changes of V_m and of hepatocyte intracellular water volume are determined in both control and Ba^{2+} treated cells. The data in Table 3 clearly shows that 2 mM Ba^{2+} partially inhibits the hyperpolarization of V_m induced by hypotonic shock. However, it seems likely that Ba^{2+} has no effect on the hypotonic shock induced change of intracellular water volume (Table 3; Fig. 10). This result is not consistent with those reported by Khalbuss et al (Khalbuss and Wondergem, 1990) in mouse liver slices and by Corasanti et al (Corasanti, Gleeson, and Boyer, 1990) in isolated rat hepatocytes. Both reported that hepatocytes lose their volume control capability and swell more when treated with Ba^{2+}. The reasons for the inconsistency between my result and theirs are unclear. However, it is very possible that the differences in tissue preparation and the technique used in determination of the cellular water volume are responsible for most of the disagreement. As mentioned previously, although the techniques are similar, nystatin

was used for intracellular TMA^+ loading in Khalbuss's experiment. My present experiment (data not shown) has demonstrated that nystatin causes loss of intracellular K^+ content in hepatocytes although the V_m remains constant. It is very possible that residual nystatin acts as a channel for TMA^+, which may influence the cell volume response to hyposmotic stress. As for Corasanti's experiment, isolated rat hepatocytes may respond differently to hyposmotic stress from that of hepatocytes in liver slices. The procedure of isolation, the difference in hypotonicity, and the different species could also contribute to this disagreement.

Quinine's effects on the Changes of a^i_{Cl} Induced by Osmotic Stresses

The data from Table 2 indicates that quinine inhibits the V_m changes in both hyper- and hyposmotic conditions. Meanwhile the a^i_{Cl} changes induced by osmotic stresses are also inhibited by quinine. In most cases, except the last one in Table 2, quinine inhibits the correlated changes of V_m and a^i_{Cl} induced by osmotic stresses so that the calculated E_{Cl} value based on the measured a^i_{Cl} value is still close to the measured V_m value (Table 2). These results are consistent with those obtained from the experiments where barium is used and thus support the idea that hepatocyte intracellular Cl^- is passively distributed with V_m in both iso- and hyper- or hyposmotic conditions. The exact

mechanisms for quinine's inhibitory effect on osmotic stresses induced changes in V_m may be complicated as mentioned before. But it is most likely that quinine's effect on a^i_{Cl} is also due to its effect on V_m as it has been suggested for Ba^{2+}.

Hepatocyte intracellular Cl^- Distributes Passively Based on the Changes of V_m Induced by Osmotic Stresses

In my experiment, the conclusion of passive distribution of hepatocyte intracellular Cl^- with the changes of V_m is supported by the following evidence. 1) Fig. 6A. and 6B. show clearly that the parallel changes of V_m and V_{Cl}-V_m during hyper- and hyposmotic stresses. 2) The Cl^- equilibrium potential (E_{Cl}), which was calculated from the Nernst equation based on the measured Cl^- activities, decreased with hyperosmotic stress from -38 ± 0.3 mV to -19 ± 2 mV; whereas, hyposmotic stress increased E_{Cl} from -26 ± 2 mV to -46 ± 3 mV. These values for E_{Cl} agreed well with the corresponding control, hyperosmotic, and hyposmotic values for V_m (Table 1). The differences between E_{Cl} and V_m for each group were well within experimental error set by the 4.2 mM detection limit for Cl^--sensitive microelectrodes. 3) After plotting the log a^i_{Cl} as a function of V_m, as it was shown in Fig. 7 and Fig. 8, the Nerstian slop (zF/RTln10) of -0.0164 fell within the 95% confidence limits of the computed regression coefficients for data shown in both

figures (Fig. 7, Fig. 8). 4) Corasanti et al. showed that after exposure to hypotonic (160 mOsm) buffer, isolated rat hepatocytes swell and then shrink back toward a steady-state volume of 1.16 times control within 1 min (Corasanti, Gleeson, and Boyer, 1990). It is obvious that the small diluting effect of cell swelling cannot account for the large percentage change of intracellular Cl^- activity during hyposmotic stress in my experiments. 5) In most cases, whenever the osmotic stress induced change of V_m is inhibited by either Ba^{2+} or quinine, the change of a^i_{Cl} is also inhibited to a corresponding degree. Thus, the calculated E_{Cl} still matches well the measured V_m (Table 2).

Khalbuss and Wondergem have shown that mouse hepatocytes lose their volume regulatory capability in hyposmotic medium and swell more when the V_m change was blocked by Ba^{2+} (Khalbuss and Wondergem, 1990). In contrast, my present findings suggest that when Ba^{2+} was present, hepatocytes swell to the similar degree as in control condition during hyposmotic stress (Table 3, Fig. 10). If the change of a^i_{Cl} were caused by the diluting effect of cell swelling, then a similar or larger than control change in a^i_{Cl} should have been seen in the presence of Ba^{2+} in my experiment. Instead, what I really see here is a much less change of a^i_{Cl} in the presence of Ba^{2+} or quinine during hyposmotic stress. So, it is obvious that the osmotic stress-induced changes in a^i_{Cl} can not be

explained by cell volume change in both control and experimental conditions.

These findings confirm and extend previous reports that hepatocyte Cl^- distributes passively and is in electrochemical equilibrium with V_m. I have shown further that this passive distribution of a^i_{Cl} holds true under conditions of hyper- and hyposmotic stresses.

Hepatocytes exhibit high Cl^- conductance of the plasma membrane (Moule and McGivan, 1990). This Cl^- conductance becomes even higher when hepatocytes are challenged by hyposmotic stress (Haddad, Beck, Boyer, and Graf, 1991). The fractional release of ^{36}Cl from isolated perfused rat liver increases by a factor of 1.69 peak value at 3-4 minutes after the onset of hypotonic stress, and then it returns to basal level within 4-5 min. The results of Haddad et al (1991) suggest that increased Cl^- efflux is a short term volume regulatory event during the RVD in rat hepatocytes. The time course of the V_m change obtained in my experiments is consistent with the time course of the change in Cl^- efflux in their experiments (Fig. 6). However, the V_m response to osmotic stress in cultured rat hepatocytes is transient (Haddad, Beck, Boyer, and Graf, 1991) and not sustained as shown here for mouse hepatocytes. Osmotic stress-induced changes in mouse hepatocyte V_m are sustained in spite of the apparent cell volume regulation (Khalbuss and Wondergem, 1990). Either mouse hepatocyte

volume regulation is incomplete or volume changes per se do not effect changes in membrane g_K and V_m. Here, osmotic stress may effect changes in intracellular mediators that modulate membrane g_K and lead to sustained V_m changes (Khalbuss and Wondergem, 1991); alternatively, applied osmotic stress may directly affect K^+ channel conductance (Zimmerberg, Bezanilla, and Parsegian, 1990).

We attribute the difference between the V_m measured in normal Krebs solution (-39 mV) and that measured in modified Krebs solution (-28 mV), in this experiment, to the partial replacement of NaCl with sucrose. We previously reported no significant effect of Cl^--free medium on the V_m, where NaCl was replaced by Na-gluconate (Wang and Wondergem, 1991). I conclude, therefore, that extracellular Na^+ is necessary for maintaining the normal V_m in hepatocytes. Fitz et al. (1989) observed that the V_m of isolated hepatocytes depolarizes on removal of HCO_3^- from the medium and hyperpolarizes in the presence of supraphysiological HCO_3^- concentrations. Acute replacement of Na^+ by choline also depolarizes hepatocytes. This depends on bicarbonate and is inhibited by DIDS but not by amiloride (Fitz, Perisco, and Scharschmidt, 1989). These results show that an electrogenic Na^+-HCO_3^- symport transfer net charge into hepatocytes. Others report a ratio of 1:3 for Na^+-HCO_3^- symport (Yoshitomi, Burckhardt, and Fromter, 1985). Thus, the Na^+-HCO_3^- symport may contribute directly to the V_m in

hepatocytes (Fitz, Perisco, and Scharschmidt, 1989). An alternative interpretation to explain the role of extracellular Na^+ in maintaining the normal V_m in hepatocytes is that Na^+ leakage into the cell may be rate-limiting for the Na^+/K^+ pump, which is stimulated continuously by intracellular Na^+. Current from the electrogenic pump could contribute directly to V_m or variation in pump activity could alter intracellular K^+ activity. There has been some studies suggest that HCO_3^--coupled Na^+ influx is a major determinant of Na^+/K^+ pump activity in rat hepatocytes (Fitz, Lidosky, Weisiger, Xie, Cochran, Grotmol, and Scharschmidt, 1991). So, it is possible that the lower resting V_m observed in my experiment where external Na^+ was partially replaced by sucrose is due to the combination of the two mechanisms mentioned above.

Taken together, my present experiments demonstrate that the hepatocyte V_m decreases while the a^i_{Cl} increases during hyperosmotic stress, and that the hepatocyte V_m increases while the a^i_{Cl} decreases during hypotonic stress. The small diluting effect of cell swelling cannot account for the large percent change in intracellular Cl^- activity during hypotonic stress. Ba^{2+} or quinine blocks the hyposmotic stress-induced change of V_m and to a similar degree the corresponding change of a^i_{Cl}. Within experimental error, osmotic stresses produce equivalent changes in V_m and E_{Cl}. Thus, my results strongly suggest that hepatocyte

intracellular Cl^- distributes passively during osmotic stresses. This phenomenon is based on the fact that hepatocyte membrane Cl^- conductance is very high. For hypotonic shock, it is likely that cell swelling-induced hyperpolarization of V_m provides an electromotive force for the efflux of intracellular Cl^-. This, in turn, contributes to the RVD mechanism in mouse hepatocytes. The a^i_{Cl} also remains passively distributed during hyperosmotic stress. This shows Cl^- conductance remains high when hepatocytes shrink. On the other hand, K^+ conductance decreases during hyperosmotic stress (Wang and Wondergem, 1991). Thus, it is likely that a high membrane Cl^- conductance is necessary for hepatocyte volume regulation and normal functioning.

CHAPTER 4

REDISTRIBUTION OF HEPATOCYTE INTRACELLULAR CHLORIDE ACTIVITY DURING HEPATIC L-ALANINE UPTAKE

INTRODUCTION

It has well been established that the majority of alanine influx in hepatocytes is Na^+-dependent and is stimulated by intracellular negativity. The ratio between cotransported Na^+ and alanine is 1 : 1 (Kristensen, 1986). The transmembrane Na^+ electrochemical gradient seems to be the exclusive driving force for intracellular alanine accumulation. At least eight distinct amino acid transport systems have been identified in hepatocytes. System A accounts for most of alanine uptake (Kilberg, 1982; Wondergem and Castillo, 1988).

This transport system has been shown to respond to a variety of physiological stimuli. Prior fasting of the animals, protein-rich diet, and certain hormones such as catecholamines, glucagon, glucocorticoids, growth hormone, insulin and thyroid hormones etc. are all known to stimulate transport by system A (Kristensen, 1986; Kilberg, 1982).

The Na^+-coupled alanine influx in hepatocytes is accompanied by changes in various other transport process. A transient depolarization followed by sustained hyperpolarization of the cell membrane was observed

(Kristensen, and Folke, 1983; Wondergem, and Castillo, 1988). In addition to the observed cell swelling, an increase in hepatocyte intracellular Na^+ accompanied by a decrease of intracellular K^+ was also reported (Kristensen, 1986).

This alanine induced increase in K^+ permeability and K^+ efflux was proposed to compensate the cell volume change and is responsible for the hyperpolarization of the hepatocyte membrane (Bear, 1990; Kristensen, 1980; Wondergem and Castillo, 1988). Alanine uptake also stimulates the Na^+/K^+ pump, which could result from the stimulating effect of increased intracellular Na^+ (Kristensen, 1986).

Relatively little is known about the response of hepatocyte intracellular Cl^- to alanine uptake. However, Cl^- distributes passively with V_m in hepatocytes (Lyall, Croxton, and Armstrong, 1987) and an inverse correlation between the Cl^- distribution and the initial rate of alanine transport has been shown by Moule and colleagues (1987).

The general model for hepatocyte alanine uptake is as follows: Na^+-coupled uptake of alanine increases intracellular Na^+ which leads to an increase in active Na^+-K^+ pumping and thus in K^+ influx; influx of alanine and cations tends to increase the cellular content of osmotically active substances, implying a tendency to water uptake; cell swelling induces an increase in the permeability of a conductive pathway for leading to net

efflux of K^+ and cellular hyperpolarization. Net efflux of K^+ prevents excessive cell volume increase during amino acid accumulation; whereas, hyperpolarization tends to support the driving force for alanine influx and anion efflux.

Because I have demonstrated previously that hepatocyte intracellular Cl^- distributes passively with V_m in both iso- and anisosmotic conditions, the present experiment is designed to test the hypothesis that L-alanine uptake induced hyperpolarization of V_m provides an electromotive force for the redistribution of hepatocyte intracellular Cl^- which may in turn contribute to cell volume regulation.

RESULTS

The Effect of L-alanine on Hepatocyte V_m

The L-alanine uptake by hepatocyte is mainly through Na^+-alanine co-transport system: system A. During L-alanine uptake, hepatocyte V_m depolarized transiently and then repolarized followed by sustained hyperpolarization. Representative traces of change in V_m induced by L-alanine are show in Fig. 11, 12, 13, and 14. In 37 measurements, the V_m hyperpolarized by 18%, from -33 ± 1 to -38 ± 1 mV when liver slices were perfused with Krebs physiological solution containing 20 mM L-alanine for 5 to 8 min.

The Effect of L-alanine on Hepatocyte a^i_{Na}

To test the assumption that hepatocyte intracellular Na^+ content increases during L-alanine uptake, a^i_{Na} was measured with double-barreled microelectrodes. In 9 measurements, 20 mM L-alanine induced a 19% hyperpolarization of V_m, from -31 ± 1 to -36 ± 1 mV and a 30% increase in intracellular Na^+ activity, from 19 ± 2 to 25 ± 3 mM (Table 4). A representative trace for Na^+ measurement from this experiment is shown in Fig. 11. This result supports the concept that L-alanine intake in hepatocytes is accomplished by Na^+-dependent amino acid cotransport system.

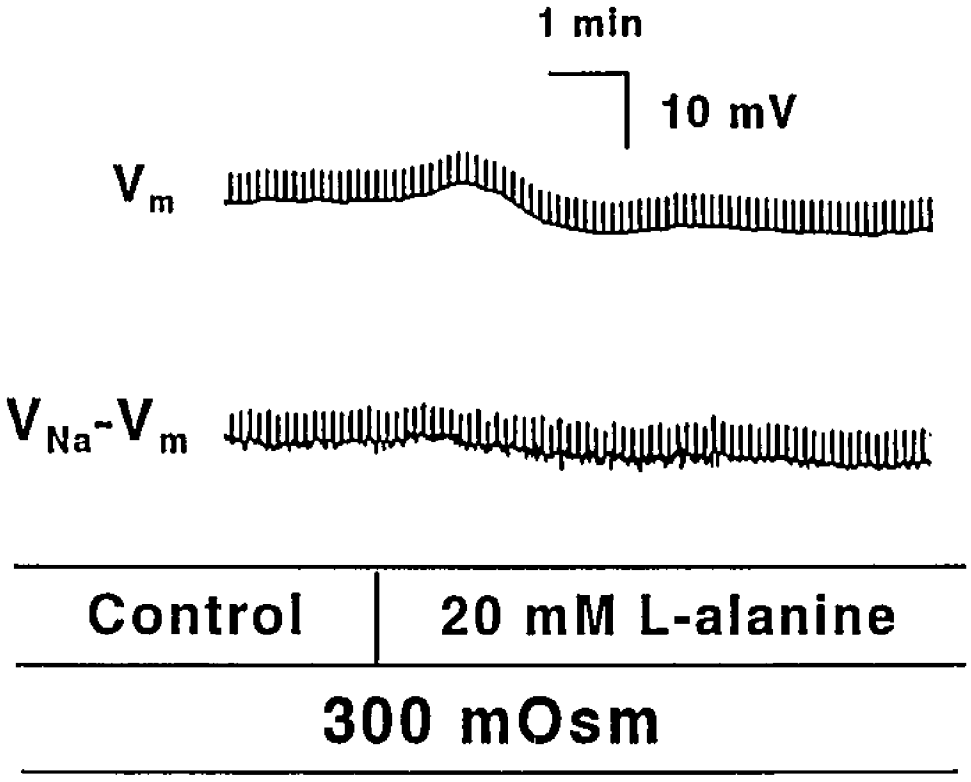

Fig. 11. Representative intracellular recordings of time-dependent changes in hepatocyte V_m and V_{Na}-V_m in response to 20 mM L-alanine. Increased electronegativity is downward in both traces. The downward deflection of V_{Na}-V_m indicates the increase of a^i_{Na}.

Table 4. The effects of L-alanine (20 mM) on the hepatocyte V_m, a^i_{Na}, a^i_K, a^i_{Cl}, a^i_T, and intracellular water volume

	N	V^c_m (-mV)	V^e_m (-mV)	V^e_m/V^c_m	$V^e_m-V^c_m$ (-mV)	a^c_X (mM)	a^e_X (mM)	a^e_X/a^c_X	a^e_X/a^c_X-1
Na^+	9	31±1	36±1*	1.19±0.04	4±1	19±2	25±3‡	1.30±0.07	
K^+	12	34±1	38±1*	1.14±0.02	5±1	83±3	79±4	0.95±0.02	
Cl^-	7	36±1	45±2*	1.24±0.03	9±1	20±2	12±2*	0.62±0.05	
TMA^+	9	31±1	37±2*	1.20±0.03	6±1	7±1	6±1†	0.9±0.03	0.12±0.03

V^c_m=hepatocyte resting transmembrane potential. V^e_m=transmembrane potential in experimental solution. a^i_{Na}, a^i_K, a^i_{Cl}, a^i_T represent intracellular Na^+, K^+, Cl^- and TMA^+ activities. N is the number of measurements. a^c_X=intracellular ion (X) activity in control condition. a^e_X=intracellular ion (X) activity in experimental condition. a^e_X/a^c_X-1 represents the intracellular water volume change when intracelluar TMA^+ is used as the marker. All values are averages ± SEM. † Differs from control, $P<0.05$. ‡ Differs from control, $P< 0.01$. * Differs from control, $P<0.001$.

The Effect of L-alanine on Hepatocyte a^i_K

To test the general idea that L-alanine induces a net loss of intracellular K^+ in hepatocytes, a^i_K was also measured during L-alanine uptake. In 12 measurements, 20 mM L-alanine induced a 14% hyperpolarization of V_m, from -34 ± 1 mV to -38 ± 1 mV and a 5% decrease in intracellular K^+ activity, from 83 ± 3 to 79 ± 4 mM (Table 4). A representative trace for K^+ measurement during L-alanine uptake is shown in Fig. 12. This L-alanine-induced decrease in hepatocyte intracellular K^+ activity was not significant.

The Effect of L-alanine on Hepatocyte a^i_{Cl}

To test my hypothesis that hepatocyte intracellular Cl^- distributes passively with V_m during L-alanine uptake, both V_m and a^i_{Cl} were measured simultaneously with double-barreled microelectrodes. In 7 measurements, 20 mM L-alanine induced a 24% hyperpolarization of V_m, from -36 ± 1 mV to -45 ± 2 mV and a 38% decrease in intracellular Cl^- activity, from 20 ± 2 to 12 ± 2 mM (Table 4). A representative trace for the change of V_m and $V_{Cl} - V_m$ induced by 20 mM L-alanine is shown in Fig. 13. The parallel changes of these two traces is readily seen.

Comparing the above V_m values with the Cl^- equilibrium potential (E_{Cl}) calculated from measured a^i_{Cl} values, 20 mM L-alanine increased E_{Cl} from -36 ± 2 mV to -49 ± 4 mV. These E_{Cl} values agree well with those V_m values and thus supports

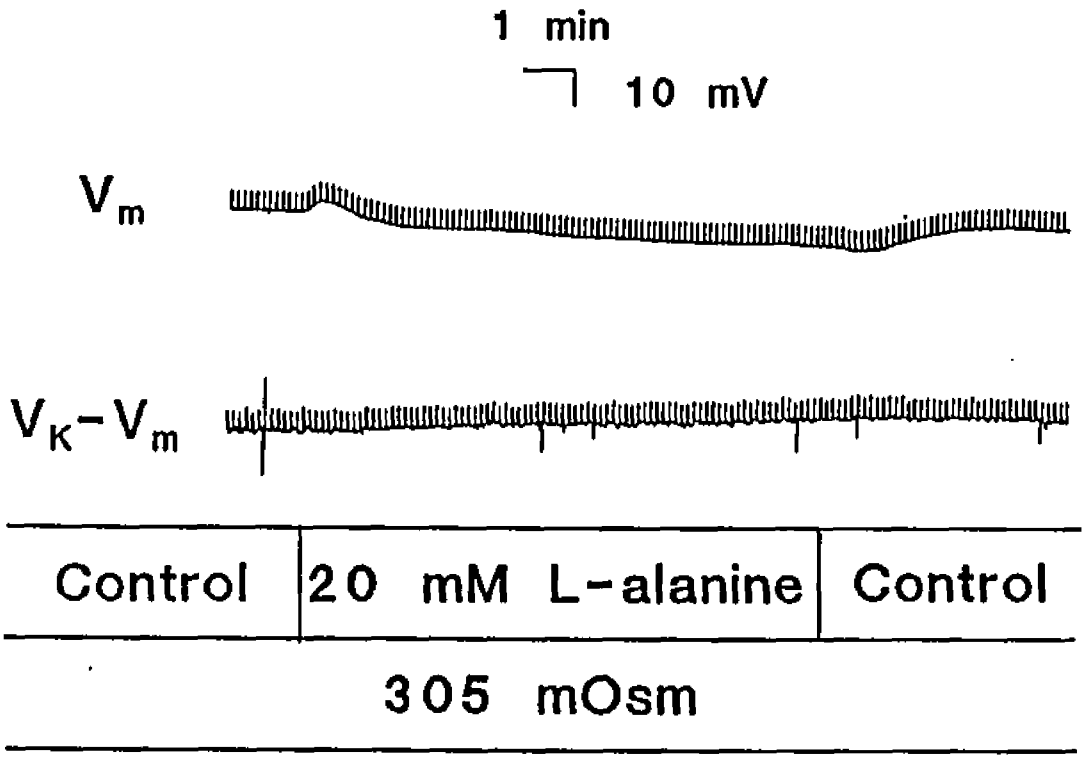

Fig. 12. Representative intracellular recordings of time-dependent changes in hepatocyte V_m and V_K-V_m in response to 20 mM L-alanine. Increased electronegativity is downward in both traces. The upward deflection of V_K-V_m indicates the decrease of a^i_K.

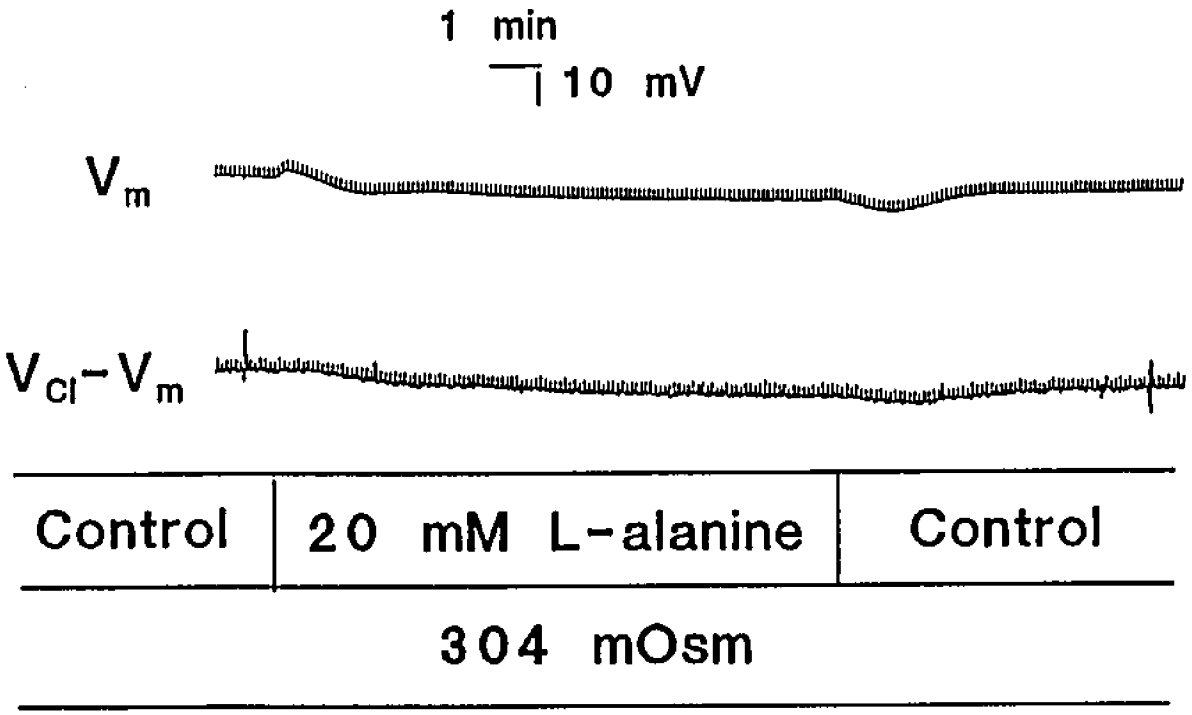

Fig. 13. Representative intracellular recordings of time-dependent changes in hepatocyte V_m and $V_{Cl}-V_m$ in response to 20 mM L-alanine. Increased electronegativity is downward in both traces. The downward deflection of $V_{Cl}-V_m$ indicates the decrease of a^i_{Cl}.

my hypothesis.

The Effect of L-alanine on Hepatocyte Intracellular Water Volume

To determine whether L-alanine-induced changes in intracellular ionic activities resulted from changes in cell water volume, the change in hepatocyte intracellular water volume was measured by the TMA^+ loading technique. In the 9 measurements after loading hepatocyte with TMA^+, 20 mM L-alanine caused a 20% hyperpolarization of the V_m, from -31 ± 1 mV to -37 ± 2 mV and a 10% decrease in a^i_{TMA}, which accounts for 12% increase in hepatocyte intracellular water volume (Table 4). A representative trace for the change of V_m and $V_{TMA} - V_m$ induced by 20 mM L-alanine is shown in Fig. 14. This data shows that hepatocytes swell during L-alanine uptake.

The Effects of Barium on L-alanine Induced Changes of V_m and a^i_{Cl}

The hyperpolarized V_m induced by L-alanine was proposed as the result of increasing in K^+ conductance, G_K, of the membrane (Kristensen and Folke, 1984). Thus barium, a well known K^+ channel blocker, should be able to block the hyperpolarized V_m, if the change of V_m is due to the increase in G_K. To test further the relationship between the change of V_m and that of a^i_{Cl}, V_m and a^i_{Cl} were measured

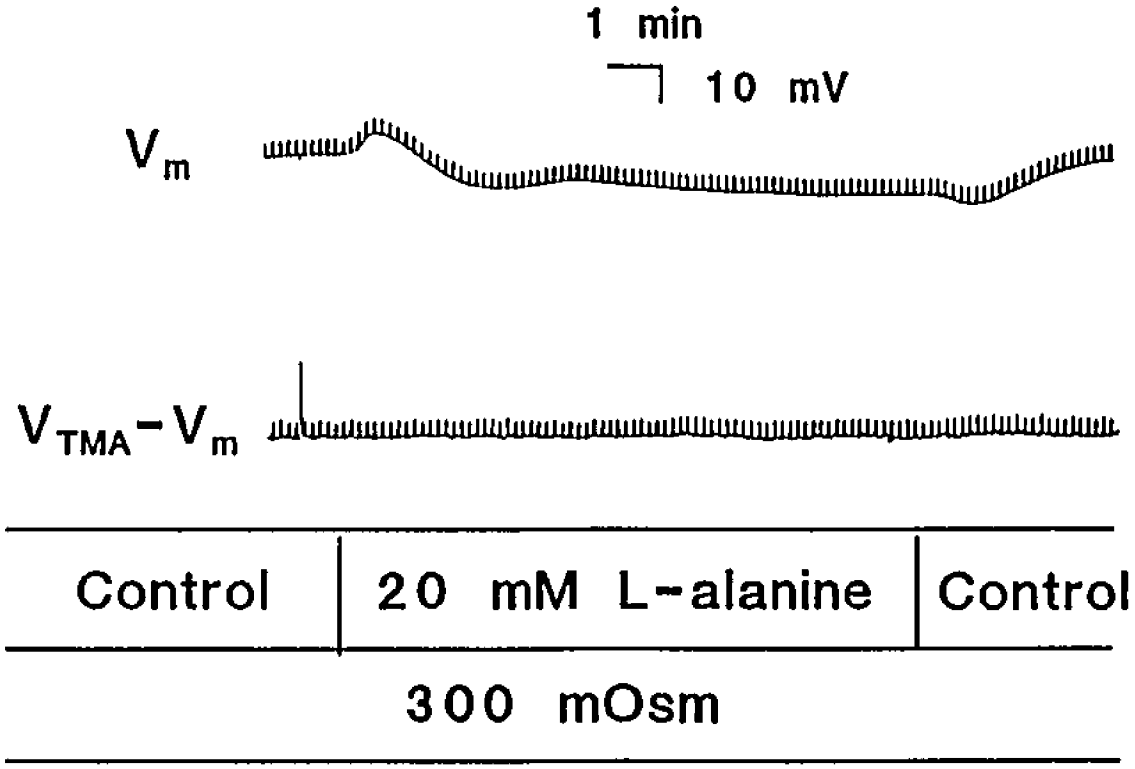

Fig. 14. Representative intracellular recordings of time-dependent changes in hepatocyte V_m and $V_{TMA}-V_m$ in response to 20 mM L-alanine. Increased electronegativity is downward in both traces. Upward deflection of $V_{TMA}-V_m$ indicates cell swelling while downward deflection represents cell shrinkage.

simultaneously when 2 mM Ba^{2+} was present in the perfusate during L-alanine uptake. Compared with the normal condition, L-alanine induced hyperpolarization of V_m was completely abolished by 2 mM barium (n = 8), and the decrease in a^i_{Cl} was also entirely blocked during L-alanine uptake. The V_m depolarized 2 mV more and did not undergo to hyperpolarization in the presence of barium (Fig. 15. Table 5). These results strongly support my hypothesis that Cl^- passively distributes with hepatocyte V_m change induced by L-alanine.

Comparing the V_m values in Table 5. with the Cl^- equilibrium potential (E_{Cl}) calculated from measured a^i_{Cl} values, the E_{Cl} during 20 mM L-alanine uptake in the presence of 2 mM Ba^{2+} changed from -33 ± 2 mV to -32 ± 3 mV which were identical to the V_m values shown in Table 5.

The Effect of Ouabain on L-alanine Induced Changes of a^i_K

Ouabain was used to determine the role of Na^+/K^+ pump in the process of L-alanine uptake induced change in hepatocyte V_m. After perfusing the liver slices with Krebs physiological solution containing 1 mM ouabain, 20 mM L-alanine induced a 7% hyperpolarization of the V_m only, from -31 ± 2 to -33 ± 3 mV but a 13% decrease in a^i_K, from 61 ± 3 to 54 ± 5 mM. This percentage of decrease in a^i_K is much higher than that of untreated liver slices (Table 6).

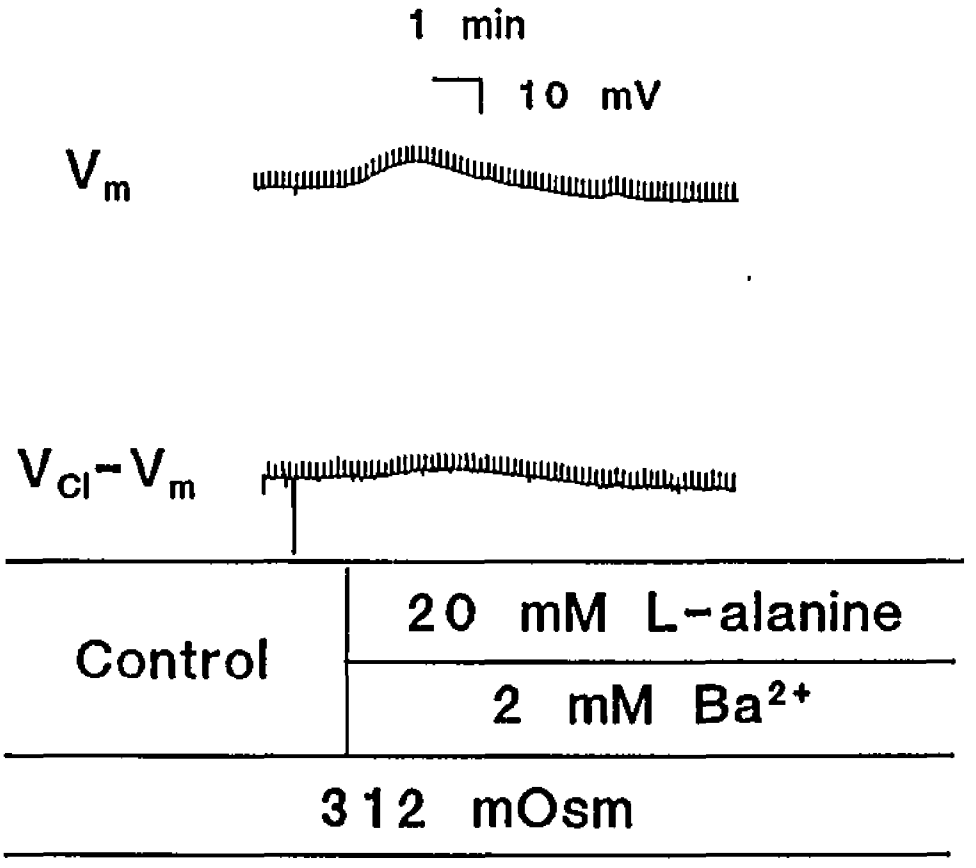

Fig. 15. Representative intracellular recordings show the coincident inhibitory effects of Ba_{2+} (2 mM) on the changes in hepatocyte V_m and $V_{Cl}-V_m$ induced by 20 mM L-alanine.

Table 5. The effect of Ba^{2+} on the L-alanine induced changes of V_m and a^i_{Cl}

	V_m^c (-mV)	V_m^e (-mV)	V_m^e/V_m^c	a^c_{Cl} (mM)	a^e_{Cl} (mM)	a^e_{Cl}/a^c_{Cl}
Untreated	36±1	45±2*	1.24±0.03	20±2	12±2*	0.62±0.05
Ba^{2+} (2mM)	33±1	32±1	0.98±0.05	22±2	24±3	1.07±0.06

V^c_m=hepatocyte resting transmembrane potential. V^e_m=transmembrane potential in experimental solution. a^c_{Cl}=intracellular Cl^- activity in control condition. a^e_{Cl}=intracellular Cl^- activity in experimental condition. All values are averages ± S.E.. * Differs from control, $P<0.001$.

Table 6. The effect of ouabain (1 mM) on the L-alanine induced changes of V_m and a^i_K

	V_m^c (-mV)	V_m^c (-mV)	V_m^c/V_m^c	a^c_K (mM)	a^c_K (mM)	a^c_K/a^c_K
Untreated	34±1	38±1*	1.14±0.02	83±3	79±4	0.95±0.02
Ouabain	31±2	33±3	1.07±0.04	61±3	54±5†	0.87±0.04

V^c_m=hepatocyte resting transmembrane potential. V^e_m=transmembrane potential in experimental solution. a^c_K=intracellular K^+ activity in control condition. a^e_K=intracellular K^+ activity in experimental condition. All values are averages ± SEM. † Differs from control, $P<0.05$. * Differs from control, $P<0.001$.

DISCUSSION

Hepatocyte V_m Depolarizes First and then Hyperpolarizes During L-alanine Uptake

The V_m traces shown in Fig. 11, 12, 13, and 14. indicate hepatocyte V_m depolarizes first and then undergoes continuous hyperpolarization during L-alanine uptake. This change of V_m is reproducible, and it is consistent with reports from others (Folke and Paloheimo, 1975; Wondergem and Castillo, 1988).

L-alanine uptake in hepatocyte is primarily through a Na^+-alanine cotransport system. Studies in isolated hepatocytes and in rat liver plasma membrane vesicles strongly suggested that the influx of alanine across the rat liver cell membrane occurs by an electrogenic 1:1 cotransport of Na^+ and alanine, and that the concentrative cellular alanine uptake is secondary to a coupled flow of Na^+ entering the cell down its electrochemical gradient (Kristensen, 1986). Addition of alanine to the extracellular medium increases the influx of Na^+ and leads to an increase in the cellular Na^+ concentration (Kristensen, 1986). Meanwhile, a transient depolarization of the cell membrane in isolated perfused liver is observed (Folke and Paloheimo, 1975). L-alanine was also found to cause hepatocyte depolarization in the skate Raja erinacea and this effect was dependent on external Na^+ (Ballatori,

Wondergem, and Boyer, 1988). Based on all these observations and on the fact that L-alanine also induced an increase in a^{i}_{Na} in my experiment (Table 4), I am confident in concluding that the initial depolarization in V_m observed in my experiment most likely results from the influx of Na^+ during L-alanine uptake.

In my experiment, administration of 2 mM Ba^{2+} entirely abolished the L-alanine-induced hyperpolarization (Table 5, Fig. 15). When the Na^+/K^+ pump was inhibited by 1 mM ouabain, the L-alanine induced hyperpolarization was blocked by 50% (Table 6). These data support the idea that the observed hyperpolarization of hepatocyte V_m right after the transient depolarization is caused primarily by the combination of: 1) the increased K^+ permeability of the membrane and 2) the activation of the Na^+/K^+ pump during L-alanine uptake. The fact that Ba^{2+} entirely abolished the L-alanine-induced hyperpolarization of V_m suggests the following possibilities: 1) L-alanine induced increase in K^+ conductance predominates the hyperpolarization of the cell membrane and 2) in addition to block the K^+ channel, Ba^{2+} may inactivate the Na^+/K^+ pump indirectly by blocking the efflux of intracellular K^+.

The L-alanine-induced increase of membrane K^+ permeability and activation of Na^+/K^+ pump have been demonstrated in hepatocytes by different laboratories (Bakker-Grunwald, 1983; Kristensen and Folke, 1984). The

increased K^+ permeability was demonstrated by a marked increased rate of K^+ efflux, and the activated Na^+/K^+ pump was confirmed by an increased initial rate of ouabain-sensitive ^{42}K uptake during L-alanine uptake (Kristensen, 1980).

The physiological function of the V_m hyperpolarization during L-alanine uptake has been proposed to be essential for supporting the Na^+ electrochemical gradient across the cell membrane during the electrogenic co-transport of Na^+ and amino acids (Kristensen, 1980). In present study, I proposed that the hyperpolarized V_m might be important for RVD because Cl^- distributes passively with V_m in hepatocytes.

Intracellular Water Volume Increases During L-alanine Uptake

The data from Table 4 indicates hepatocyte intracellular water volume increases by 12% during L-alanine uptake. This value is identical to that reported by Kristensen in isolated rat hepatocytes (Kristensen, 1986). However, since their data suggests that cellular water volume reaches a new increased steady level after 20 min of alanine uptake, I cannot exclude the possibility that mouse hepatocytes in liver slice will swell a little further after prolonged alanine perfusion. However, my measurements are valid because almost all of my intracellular recordings are made within the same time course range.

The Effect of L-alanine on Hepatocyte Intracellular K^+ activity

In the 12 measurements from different cells of my experiment, hepatocyte intracellular K^+ activity decreases by 5% from 83 ± 3 to 79 ± 4 mM while the V_m hyperpolarizes by 14% from -34 ± 1 to -38 ± 1 mV within 5-6 min during L-alanine uptake (Table 4, Fig. 12). The decreased a^i_K is not statistically significant different from that of control value. Actually, considering the cell volume change during alanine uptake, my data does not show any net loss of intracellular K^+ (Table 4). Since L-alanine-induced increase in unidirectional K^+ efflux has been demonstrated by several groups (Kristensen, 1980; Cohen and Lechene, 1990), it is possible that the time course (5-6 min) used for K^+ measurement in my experiment is not long enough to create a significant net loss of intracellular K^+ content. Kristensen reported, in isolated rat hepatocytes, that intracellular K^+ content starts to decrease right after the onset of the alanine administration and this decrease in cellular K^+ reaches a new stable minimum level after 20 mins (Kristensen, 1986). His study supports the above explanation. However, my result is consistent with the data from the study of Cohen and Lechene (1990). In their experiment, a 15 min exposure to 10 mM alanine did not alter the intracellular K^+ content in primary cultured rat hepatocytes. Meanwhile, an increase in Na^+/K^+ pump rate, an

increase in K^+ efflux, and an increase in the initial rate of K^+ loss after Na^+/K^+ pump inhibition were also reported (Cohen, B. J., and C. Lechene, 1990). Thus, they attribute the failure of alanine to reduce intracellular K^+ content to stimulation of Na^+/K^+ pump.

In order to test the same mechanism in my experiment, intracellular K^+ measurement is repeated in the presence of 1 mM ouabain during alanine uptake. The results are presented by Table 6. The data in Table 6 confirms the assumed Na^+/K^+ pump activating mechanism by showing that alanine transport significantly decreases intracellular K^+ activity after inhibition of the pump by ouabain. Actually, inhibiting the Na^+/K^+ pump by ouabain causes cellular K^+ loss and equivalent Na^+ gain without swelling in liver slices (Macknight, Pilgrim, and Robinson, 1974; McLaughlin, 1973). This will decrease the Na^+ transmembrane electrochemical gradient and relatively less Na^+ will enter the cell with alanine in the presence of ouabain. In this case, my data should be an underestimate for the Na^+/K^+ pump activation during L-alanine uptake. In the other words, L-alanine should produce an even bigger decrease of intracellular K^+ activity than has been shown in Table 6 in the presence of ouabain, if the intracellular Na^+ activity were normal. Thus, my present study strongly suggests that L-alanine uptake stimulates both intracellular K^+ efflux and Na^+/K^+ pump activity simultaneously, so that the

intracellular K^+ content remains constant in mouse hepatocytes. The inhibitory effect of ouabain on the Na^+/K^+ pump in my experiment has been confirmed by the fact that the a^i_K in ouabain treated group before L-alanine uptake is significantly lower than that of untreated group. Interestingly, in addition to blocking the activity of Na^+/K^+ pump, ouabain also blocks the alanine induced hyperpolarization of V_m. I have already shown that ouabain has no effect on hypotonic shock induced hyperpolarization of V_m (see next chapter, Table 10), this may suggests that the mechanisms of hyperpolarization of V_m between alanine uptake and hypotonic shock are different. In the case of alanine uptake, both the increased K^+ conductance and the activated Na^+/K^+ pump activity contribute to the V_m hyperpolarization, while only the increased K^+ conductance is responsible for the V_m hyperpolarization during hypotonic stress. Since the main difference between these two conditions is the intracellular Na^+ content, it is very likely that an increase in intracellular Na^+ content is the major stimulation for efflux of K^+ and the Na^+/K^+ pump activity.

Hepatocyte intracellular Cl^- Distributes Passively Based on the Change of V_m Induced by L-alanine Uptake

Since hepatocyte intracellular Cl^- is passively distributed with V_m during hypotonic shock and the mechanism

of RVD during hyposmotic stress is similar to that during L-alanine uptake, it is very likely that hepatocyte intracellular accumulation of alanine will be accompanied by a decrease of intracellular Cl^- content because it has been known that alanine induces a hyperpolarization of V_m in hepatocytes (Fitz and Scharschmidt, 1987; Wondergem and Castillo, 1988). In fact, Moule and colleagues (1987) had found an inverse correlation between the Cl^- distribution (expressed as the ratio of internal to external concentrations) and the initial rate of alanine transport in isolated rat hepatocytes (Moule, Bradford, and McGivan, 1987).

To test the hypothesis that hepatocyte intracellular Cl^- distributes passively with the hyperpolarized V_m induced by L-alanine, hepatocyte V_m and a^i_{Cl} were measured simultaneously and continuously with double barreled Cl^--sensitive microelectrodes during L-alanine uptake. My recordings show clearly that L-alanine induces a transient depolarization followed by a sustained hyperpolarization of V_m. This hyperpolarized V_m is accompanied by a parallel change of V_{Cl}-V_m, which is proportional to the decreasing of intracellular Cl^- activity, a^i_{Cl} (Fig. 13). After 7 individual measurements in 7 different cells, L-alanine (20 mM) induces a 24% hyperpolarization of V_m from -36 ± 1 to -45 ± 2 mV. Meanwhile, the intracellular Cl^- activity decreases by 38% from 20 ± 2 to 12 ± 2 mM in 5 to 8 mins

(Table 4).

Hepatocytes swell during alanine uptake. Rat hepatocyte intracellular water volume increases by 12% after 30-min incubation in a 280 mOsmol buffer containing 10 mM alanine (Kristensen, 1986). Using intracellular TMA^+ as the probe, my experimental results also indicate a 12% increase in mouse hepatocyte intracellular water volume within the same time course that a^i_{Cl} was measured during L-alanine uptake (Table 4). So, it is very clear that the 38% decrease in a^i_{Cl} cannot be explained by the 12% diluting effect of hepatocyte swelling during L-alanine uptake. On the other hand, if the alanine induced hyperpolarization of V_m is inhibited by Ba^{2+}, which is a well known K^+ channel blocker, the decrease of a^i_{Cl} induced by alanine is also blocked (Table 5). Thus, my data strongly suggests that mouse hepatocyte intracellular Cl^- activity passively distributes with the L-alanine induced hyperpolarization of V_m.

We attribute the lack of corresponding response of V_{Cl}-V_m during the initial transient L-alanine-induced depolarization of V_m to the diffusion delay of Cl^- across the cell membrane. This conclusion is supported by the following observations: 1) The diffusion delay of intracellular Cl^- following the change of V_m can be seen relatively clearly when the V_m change is fast (Fig. 6 A. and B.). 2) The V_{Cl}-V_m has a tendency to follow the

depolarization of V_m during alanine uptake shown in Fig. 15 where the V_m repolarization-hyperpolarization is inhibited by Ba^{2+}. In this case, alanine induced increase in K^+ efflux is blocked, the time course for the depolarization of V_m is protracted and the magnitude larger. A corresponding upward deflection of V_{Cl}-V_m trace, indicating an increase of a^i_{Cl}, can be seen.

Hepatocyte membrane Cl^- permeability is high. In perfused rat liver, the calculated permeability coefficients for K^+ is = 7.6 * 10^{-6} cm/sec while the P_{Cl} = 12.3 * 10^{-6} cm/sec, and the G_{Cl} is three times greater than G_K (Claret and Mazet, 1972). Cl^- was found to cross the liver cell membrane relatively rapidly (Moule, Bradford, and McGivan, 1987). Using the similar technique as ours in intact rat liver, Fitz et al indicated that intracellular Cl^- activities are close to the level predicted for passive distribution under basal conditions and after hyperpolarization of the V_m by alanine (Fitz and Scharschmidt, 1987). But their conclusions are weakened by the lack of continuous intracellular recordings of V_m and V_{Cl}-V_m during the whole period of alanine uptake process. In other words, their intracellular recordings of a^i_{Cl} before and after alanine uptake were not from the same cell.

Our present study, therefore, is the first intracellular measurement showing directly and simultaneously the continuous intracellular changes of V_m

and a^i_{Cl} during L-alanine uptake in mouse hepatocytes. In addition, my data also demonstrated that the L-alanine induced decrease of a^i_{Cl} is due to the increased V_m instead of cell swelling. There are various possibilities regarding the physiological significance of this phenomenon: 1) The decrease of intracellular Cl^- activity will definitely facilitate the RVD process during alanine uptake. 2) The alanine induced change in a^i_{Cl} may play a role in hepatic bile flow, bile acid excretion, and exocytosis because hepatocyte swelling and RVD has been found to stimulate these events (Bruck, Haddad, Graf, and Boyer, 1992) and Cl^- is the predominant anion in bile (Fitz and Scharschmidt, 1987). 3) The change of hepatocyte intracellular Cl^- content caused by alanine might serve as a second messenger to mediate certain intracellular biochemical processes in regulating new carrier protein synthesis and/or insertion of the new carrier molecules into hepatocyte membrane, because the hormonal and adaptive regulation of system A is considered to be due, in part, to the synthesis and insertion of new carrier molecules into the cell membrane (Moule, Bradford, and McGivan, 1987). Cl^- has been found to inhibit IP_3 induced Ca^{2+} release from endoplasmic reticulum in rat hepatocytes (Joseph and Williamson 1986). Cl^- was also found to increase the affinity of G protein for GTP at modest concentrations (Higashijima, Ferguson, and Sternweis, 1987). In snail ganglia, Cl^- stimulates

adenylate cyclase activity in a GTP-dependent manner (Deterre, Gozlan, and Bockaert, 1983). All these studies have drawn attention to the change of intracellular Cl^- could serve as a secondory messenger itself.

CHAPTER 5

MOUSE HEPATOCYTE INTRACELLULAR WATER VOLUME RESPONSE AND INTRACELLULAR POTASSIUM ACTIVITY CHANGES DURING SHORT-TERM HYPOTONIC SHOCK

INTRODUCTION

The normal functioning of hepatocytes requires a stable intracellular ionic and pH environment. Membrane transport, cellular metabolism and bile secretion constantly change hepatocyte intracellular organic solute concentration which is accompanied by the movements of water and inorganic ions across the cell membrane. Examples include hepatocyte intracellular accumulation of amino acids through Na^+-dependent amino acid cotransport systems and hormone stimulated glycogenolysis. Thus, hepatocytes regulate their volume even when the extracellular osmolality remains constant, although that is not always the case because the intestinal absorption can change the osmolality of the portal blood. In fact, cell swelling induced regulatory volume decrease (RVD) has been well confirmed during alanine uptake in hepatocyte (Kristensen, 1986).

The general pattern of RVD in hepatocyte consists of cellular swelling induced loss of intracellular K^+ accompanied by anions and water follows. Hypotonic stress also induces a hyperpolarization of the hepatocyte

transmembrane potential, V_m, which is caused by an increase in cell membrane K^+ conductance, G_K. Several reports have suggested that RVD in rat hepatocytes is mediated by efflux of intracellular K^+ (Haddad and Graf, 1989; Haussinger, Stehle, and Lang, 1990; Corasanti, Gleeson, and Boyer, 1990).

Intracellular K^+ is vital to the cell, and the level of decrease in intracellular K^+ has been used as an indicator of toxicity in hepatocytes (Smith, Fisher, Shubat, Gandolfi, Krumdieck, and Brendel, 1987). From this point of view, any loss of cellular K^+ could impair cellular metabolism.

Using an electrophysiological technique, the present study is designed to evaluate: (1) to what degree does the hepatocyte intracellular K^+ decrease during hypotonic stress; (2) what causes this K^+ decrease; (3) what is the relationship between the change of intracellular K^+ and that of cellular water volume.

RESULTS

The Effect of Hyposmotic Stress on a^i_K

Reducing osmolality of the external medium from 300 to 250 mOsm, by reducing medium sucrose, resulted an 19-mV hyperpolarization of the hepatocyte V_m, from -27 ± 1 mV to -46 ± 1 (n = 15), over 4-5 min until a steady state was achieved (Table 7; Fig. 16A, 17A). The hyperpolarized V_m returned to control value within 3-4 min after switching back to control medium. Meanwhile, the same hypotonic shock decreased the hepatocyte a^i_K by 14% from 91 ± 4 to 78 ± 4 mM in the same cells.

The Effect of Hyposmotic Stress on a^i_{TMA} and Intracellular Water Volume

In order to test the hypotonic stress-induced hepatocyte intracellular water volume change, I monitored the intracellular TMA^+ activity, a^i_{TMA}, change induced by hypotonic stress in the hepatocytes loaded with TMA^+. Hypotonic stress of 0.82 times control induced a 12% decrease in hepatocyte a^i_{TMA}, which represents a 14% increase in hepatocyte water volume (Table 8, Fig. 16B). Thus, it is possible that the hypotonic shock induced decrease in a^i_K is due to the swelling of the cell.

Table 7. Effect of hypotonic stress on hepatocyte V_m and a^i_K (n=15)

V^c_m (-mV)	$V^{c'}_m$ (-mV)	$V^{c'}_m$ (-mV)	a^c_K (mM)	$a^{c'}_K$ (mM)	$a^{c'}_K$ (mM)	$a^{c'}_K/a^c_K$	$\pi^{c'}/\pi^c$
27±1	46±1*	26±1	91±4	78±4*	88±4‡	0.86±0.01	0.82

V^c_m=hepatocyte transmembrane potential before hypotonic stress. $V^{c'}_m$=transmembrane potential in hyposmotic experimental solution. $V^{c'}_m$=hepatocyte transmembrane potential after hypotonic stress. a^c_K=hepatocyte intracellular K^+ activity in isosmotic control solution. $a^{c'}_K$=intracellular K^+ activity in hyposmotic experimental solution. $a^{c'}_K$=hepatocyte intracellular K^+ activity after hypotonic stress. All values are averages ± S.E.; values for individual experiments are from at least three separate measurements, whereas the mean values are averages of the values from each animal. ‡ Differs from control, P<0.01. * Differs from control, P<0.001.

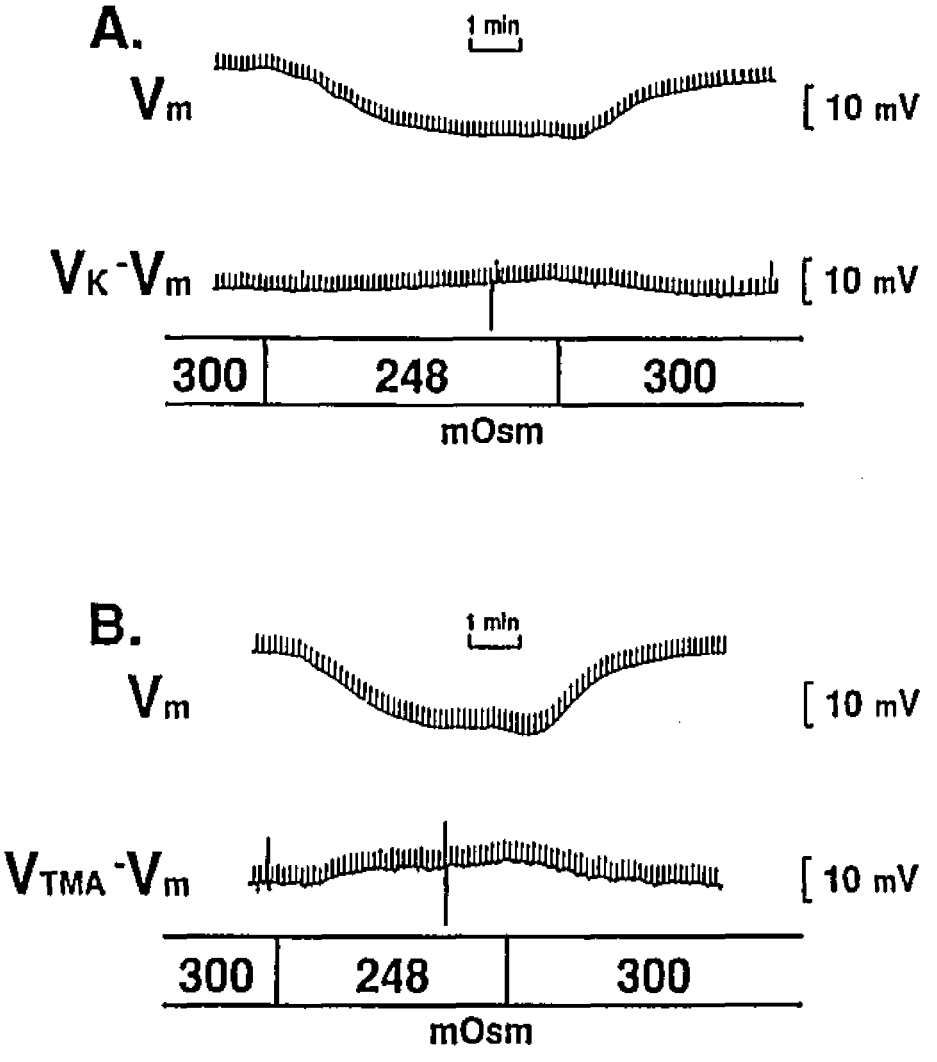

Fig. 16. Comparison of hyposmotic stress induced change of a^i_K (represented by V_K-V_m) in A. with that of a^i_{TMA} (represented by V_{TMA}-V_m) in B. Greater electronegativity is downward in all traces. The upward deflection of V_K-V_m in A. indicate the decrease of a^i_K. The upward deflection of V_{TMA}-V_m in B. indicate the decrease of a^i_{TMA}.

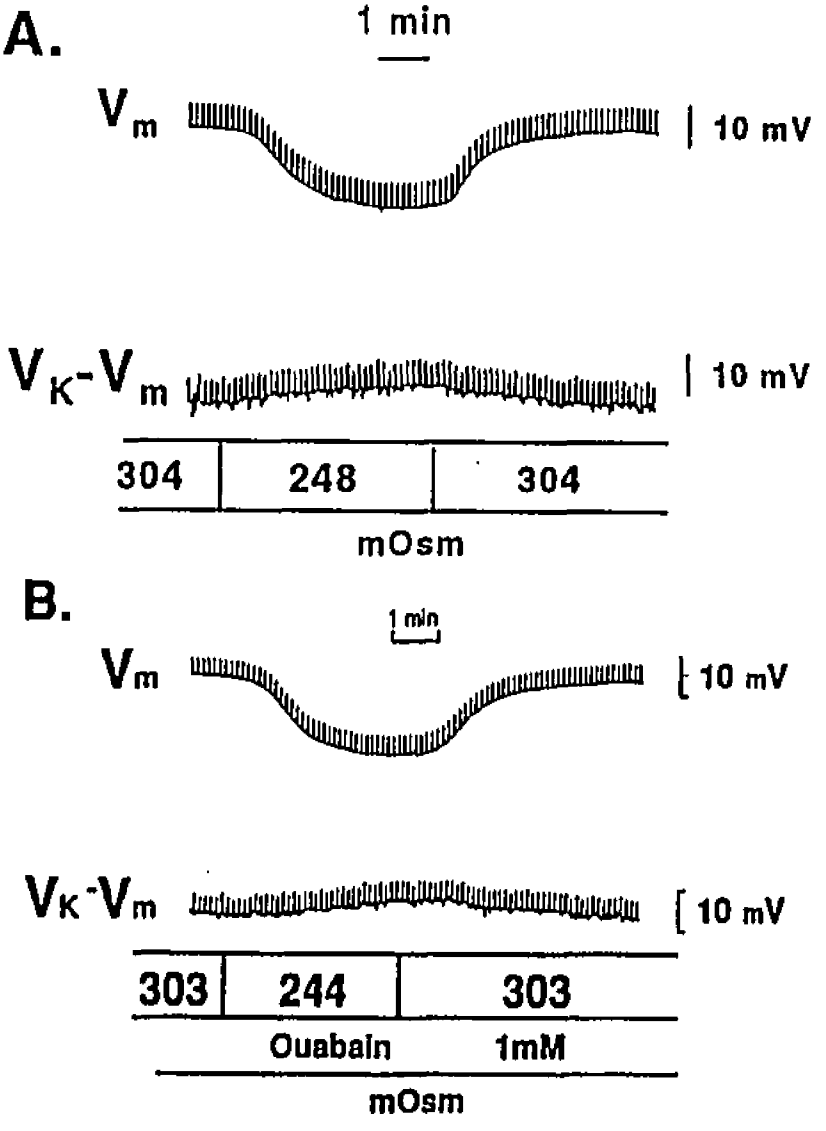

Fig. 17. Representative intracellular recordings of time-dependent changes in hepatocyte V_m and V_K-V_m during hyposmotic stress without and with ouabain (1 mM). Greater electronegativity is downward in all traces. The upward deflections of V_K-V_m in both A. and B. indicate the decrease of a^i_K.

Table 8. Effect of hypotonic stress on hepatocyte V_m and a^i_{TMA} (n = 6)

V^c_m (-mV)	V^e_m (-mV)	V^e_m/V^c_m	a^c_T (mM)	a^e_T (mM)	a^e_T/a^c_T	a^e_T/a^c_T-1	π^c/π^e
29±2	45±2*	1.57±0.07	18.2±2.9	16.0±2.4‡	0.88±0.01	0.14±0.02	0.82

V^c_m=hepatocyte transmembrane potential before hypotonic stress. V^e_m=transmembrane potential in hyposmotic experimental solution. a^c_T=hepatocyte intracellular TMA^+ activity in isosmotic control solution. a^e_T=intracellular TMA^+ activity in hyposmotic experimental solution. a^e_T/a^c_T-1 represents the intracellular water volume change when intracellular TMA^+ is used as the marker. All values are averages ± S.E.; values for individual experiments are from at least three separate measurements, whereas the mean values are averages of the values from each animal. ‡ Differs from control, P<0.01. * Differs from control, P<0.001.

Comparison Between the Hypotonic Stress Induced Changes of V_m, a^i_K, a^i_{TMA} and Cell Water Volume

To test this hypothesis, hepatocyte a^i_K, and a^i_{TMA} were measured separately in paired experiments. The results were shown in Table 9. Hypotonic shock of 0.83 times control osmolality hyperpolarized V_m by 71%, from -26 ± 0 mV to -44 ± 2 mV, and decreased the a^i_K by 15%, from 88 ± 3 to 75 ± 3 mM. After loading hepatocyte with TMA^+, a similar hypotonic stress (0.82 times control Osm) caused a 45% hyperpolarization of V_m, from -32 ± 1 to -46 ± 1 mV, and a 14% decrease in a^i_{TMA} which account for a 17% increase in intracellular water volume. One interesting point is that the intracellular water volume would increase to the same degree if the intracellular K^+ was used as the marker. These results strongly support my hypothesis that hepatocyte swelling is responsible for the hypotonic shock induced decrease in a^i_K.

The Effects of Ouabain on Hyposmotic Stress Induced Changes of V_m and a^i_K

In order to test the possibility that the activation of Na^+/K^+ pump compensates the net loss of intracellular K^+ caused by hypotonic shock-induced increasing in efflux of intracellular K^+ in my experiment, V_m and a^i_K were measured respectively in normal as well as in ouabain present condition during hypotonic shock. In this paired

Table 9. Comparison of the effect of hypotonic stress on intracellular a^i_K and a^i_{TMA} (n=3)

	V^c_m (-mV)	V^e_m (-mV)	V^e_m/V^c_m	$V^e_m-V^c_m$ (-mV)	a^c_K (mM)	a^e_K (mM)	a^e_K/a^c_K	a^c_K/a^e_K-1	π^e/π^c
K^+	26±0	44±2*	1.71±0.10	18±2	88±3	75±3‡	0.85±0.01	0.17±0.01	0.83
	V^c_m (-mV)	V^e_m (-mV)	V^e_m/V^c_m	$V^e_m-V^c_m$ (-mV)	a^c_T (mM)	a^e_T (mM)	a^e_T/a^c_T	a^c_T/a^e_T-1	π^e/π^c
TMA^+	32±1[dt]	46±1*	1.45±0.08	14±2	23±3	20±3†	0.86±0.01	0.17±0.01	0.82

V^c_m=hepatocyte transmembrane potential. V^e_m=transmembrane potential in hyposmotic experimental solution. a^c_K=hepatocyte intracellular K^+ activity in isosmotic control solution. a^e_K=intracellular K^+ activity in hyposmotic experimental solution. a^c_T=hepatocyte intracellular TMA^+ activity in isosmotic control solution. a^e_T=intracellular TMA^+ activity in hyposmotic experimental solution. a^e_T/a^c_T-1 and a^c_K/a^e_K-1 represent the relative intracellular water volume change while intracellular TMA^+ or K^+ was used as the marker. All values are averages ± S.E.; values for individual experiments are from at least three separate measurements, whereas the mean values are averages of the values from each animal. † Differs from control, $P<0.05$. ‡ Differs from control, $P<0.01$. * Differs from control, $P<0.001$. [dt] Differs from above value, $P<0.05$.

experiment, hypotonic shock (0.82 times control osmolality) induced a 82% increasein V_m, from -24 ± 1 mV to -44 ± 0 mV and a 13% decrease in a^i_K, from 81 ± 4 to 70 ± 5 mM during hypotonic shock in control condition. After exposing the liver slices to Krebs physiological solution containing 1 mM ouabain for half hour, the same hypotonic stress still caused a 82% increase in V_m, from 22 ± 2 to 44 ± 1 mV and a 13% decrease in a^i_K, from 64 ± 3 to 56 ± 3 mM.(Fig. 17, Table 10). The effect of ouabain on Na^+/K^+ pump has been indicated by the fact that the resting a^i_K value in ouabain group was significantly lower than that of untreated group. These results suggest that hypotonic shock-induced hyperpolarization of V_m and decrease of a^i_K are independent of the pump.

Table 10. Effect of ouabain (1 mM) on hypotonic stress induced changes of V_m and a^i_K (n=4)

	V^c_m (-mV)	V^e_m (-mV)	$V^{e'}_m$ (-mV)	V^e_m/V^c_m	a^c_K (mM)	a^e_K (mM)	$a^{e'}_K$ (mM)	a^e_K/a^c_K	π^e/π^c
Untreated	24±1	44±0*	25±1	1.82±0.07	81±4	70±5‡	80±4	0.87±0.02	0.82
Ouabain	22±2	40±1*^{dt}	23±2	1.82±0.09	64±3^{d*}	56±3†^{d*}	63±4^{d*}	0.87±0.02	0.82

V^c_m=hepatocyte transmembrane potential. V^e_m=transmembrane potential in hyposmotic experimental solution. $V^{e'}_m$=transmembrane potential after hyposmotic stress. a^c_K=hepatocyte intracellular K^+ activity in isosmotic control solution. a^e_K=intracellular K^+ activity in hyposmotic experimental solution. $a^{e'}_K$=intracellular K^+ activity after hyposmotic stress. All values are averages ± S.E.; values for individual experiments are from at least three separate measurements, whereas the mean values are averages of the values from each animal. † Differs from control, P<0.05. ‡ Differs from control, P<0.01. * Differs from control, P<0.001. ^{dt} Differs from untreated value, P<0.05. ^{d*} Differs from untreated value, P<0.001.

DISCUSSION

Hepatocyte Intracellular Water Volume Increases During Hyposmotic Stress

Using intracellular TMA^+ as the marker, mouse hepatocyte intracellular water volume is found to increase by 14% during 0.82 times control osmolality of hypotonic shock (Table 8, Fig. 16). This value is close to that reported by others in both isolated rat hepatocytes and mouse liver slices (Corasanti, Gleeson, and Boyer, 1990; Khalbuss and Wondergem, 1990). However, since the magnitude of hypotonicities applied in these experiments are different, this comparison is relatively meaningless. As mentioned previously, a 82% hypotonic stress would have caused a 22% increase in cellular water volume if hepatocytes respond as a perfect osmometer. Thus, the 14% increase in cell water volume measured in my experiment suggests that a cell volume control mechanism exists in hepatocyte during hypotonic stress. Corasanti et al showed that after exposure to hypotonic buffer, isolated rat hepatocytes swelled 1.44 times control volume within 30-60 s and then underwent a RVD toward a steady-state volume of 1.16 times control within 5-10 min (Corasanti, Gleeson, and Boyer, 1990).

This kind of typical RVD process has never been observed in my experiments. One of the possibilities might

be that hepatocytes in mouse liver slice need more time for the recovery of their volume during hypotonic stress. But the technique I used makes it extremely difficult to test this possibility because of the difficulty of keeping the electrode within the same cell for such a long period of time without any leakage. However, since the cell volume measured in my experiments during hypotonic stress is close to those recovered cell volume value reported by others, it is most likely that the observed different behavior of hepatocyte volume to hypotonic stress represents the different physical properties between isolated hepatocytes and cells in situ. It is not difficult to imagine that hepatocyte in situ cannot change its volume the same way as it does in isolated condition because each cell is tightly attached by several neighbors. Finally, the isolation process it self may change the property of the cell membrane.

Ouabain Has No Effect on Hyposmotic Stress Induced Change of V_m

In paired experiments, same hyposmotic stress induced a 182% hyperpolarization in both control group and the group with added ouabain (Table 10). This data suggests 1) the contribution of Na^+/K^+ pump to the hypotonic shock induced change of V_m is very small; 2) the hypotonic stress induced increase in K^+ conductance, G_K, is independent of Na^+/K^+

pump activity. My result is consistent with Cohen and Lechene's finding in which they reported that 10 mM alanine induced an increase in unidirectional K^+ efflux that is independent of Na^+/K^+ pump in primary cultured rat hepatocytes (Cohen, B. J., and C. Lechene, 1990).

The results from Table 10 also shows that 1 mM ouabain does not decrease hepatocyte resting V_m significantly after 30 min. But ouabain does exerts its effect on hepatocytes by significantly reducing the intracellular K^+ activity by 21% from 81 ± 4 to 64 ± 3 mM (Table 10). This data suggests that 1) compared to many other cell types, the immediate contribution of Na^+/K^+ pump activity to hepatocyte V_m is relatively small; 2) unlike certain other cell types, the contribution of K^+ conductance, G_K, to the hepatocyte V_m is relatively small. This is consistent with our previous study in that the transference number for K^+, which is a indication of the relative contribution of K^+ conductance to overall cell membrane conductance, is 0.20 ± 0.01 (Wang and Wondergem, 1991). These findings are also consistent with the result of Graf et al.. In their study, they reported that the immediate effect of ouabain on the negative membrane potential in isolated rat hepatocytes is to reduce it by <3 mV (Graf, Henderson, Krumpholz, and Boyer, 1987).

Hepatocyte Intracellular K^+ Decreases During Hyposmotic Stresses

In my experiment, a 0.82 times control hyposmotic stress produces a 70% hyperpolarization of V_m and a 14% decrease of hepatocyte intracellular K^+ activity in the 45 measurements from 15 animals (Table. 7. and Fig. 16A, 17A). This result is consistent with the finding of others in that a decrease of intracellular K^+ content was also found when hepatocytes were challenged with hypotonic shock (Cohen, and Lechene, 1990).

Haddad et al. (1990), by monitoring the effluent K^+ activity, have shown that initiation and termination of hypotonic stress in isolated perfused rat liver triggered a sharp transient (<1 min) net water movement into and out of the liver. In addition, hypotonic stress caused a large transient net release of hepatic K^+, whereas return to isotonicity triggered a transient net hepatic K^+ uptake. The hypotonically induced K^+ release was inhibited by barium and quinine and the net K^+ uptake after hypotonic shock was inhibited by ouabain and amiloride. Using the same methods, Haussinger et al. (1990) reported the similar results. Berthon et al. (1980) also reported a hypotonic shock induced 10% increase in hepatocyte volume triggered an transient increase in K^+ efflux rate constant. Their data suggest an increase in G_K and a net loss of intracellular K^+ during hypotonic shock induced RVD in hepatocytes.

Hypotonic stress induced intracellular loss of K^+ during RVD has been found in other cell types. In 1971, Kregenow first reported, in duck erythrocytes, that hypotonic stress increases membrane K^+ permeability, resulting in cell K^+ loss (Kregenow, 1971). Grinstein et al. (1982) reported that, in lymphocytes, hypotonic medium induced a efflux of K^+ during RVD which was independent from the conductive movement of Cl^-. Larson and Spring (1984) demonstrated, in Necturus gallbladder cells, that both movements of K^+ and Cl^- out of the cell across the basolateral membrane are required in the RVD process.

Based on my measurements, 4-5 min hypotonic shock (82% of control osmolality), causes a 14% decrease in intracellular K^+ activity. However, this decrease in a^i_K most likely results from hepatocyte swelling. Firstly, the a^i_K almost completely returns to the control level after hypotonic shock (Fig. 16; and Table 7). The 3% loss in a^i_K after hypotonic stress, $a^{c'}_K$ compared with control level a^c_K, can be satisfactorily explained by leakage of K^+ at the impalement site of the cell membrane during the recording process with microelectrodes. This is supported by the fact that the V_m measured at the same time is also 2% less then control value. Secondly, ouabain has no effects on the change of a^i_K both during and after hypotonic stress. In paired experiments, the percentage of decrease in a^i_K (12% in control and 12% in ouabain group) and percentage of recovery

in a^i_K (99% in control and 98% in ouabain group) are almost identical (Fig. 17; Table 10). This means that the assumed compensatory K^+ uptake through the activated Na^+/K^+ pump during the period of hypotonic stress can be ignored. Thirdly, in paired experiments (Table 9), the percent decrease in intracellular a^i_K and a^i_{TMA} is almost identical (15% and 14% respectively), and the increased intracellular water volume (17 ± 1%) measured in TMA loaded hepatocytes is identical with those measured in control group where intracellular K^+ was used as the probe (17 ± 1%). So, up to this point, there is no evidence of net loss of intracellular K^+ that is detectable by K^+ sensitive-microelectrode during short-term (up to 5 min) hypotonic stress in my experiments. However, my present study does not exclude the possibility of certain amount of K^+ net loss during a prolonged, higher degree of hypotonic stress.

The only direct evidence I have found so far of hepatocyte loss of intracellular K^+ in hypotonic solution comes from Cohen's report (Cohen and Lechene, 1990). In their study, rat hepatocyte intracellular K^+ fell by 18% after 15 min exposure to hypotonic solution where NaCl concentration was reduced by 57 mM. But, it is possible that at least part of this loss in K^+ could result from low extracellular Na^+ concentration in addition to the hyposmotic condition alone. HCO_3^--coupled Na^+ influx has been suggested as a major determinant of Na^+/K^+ pump

activity in rat hepatocytes (Fitz, Lidosky, Weisiger, Xie, Cochran, Grotmol, and Scharschmidt, 1991). Thus, lowering extracellular Na^+ concentration could inhibit the Na^+/K^+ pump, causing a decrease in intracellular K^+, because K^+ normally diffuses out of the cell down its electrochemical gradient. In my experiment, the higher level of a^i_K in control comparing with that in ouabain group further confirmed this phenomenon (Table 10). Other factors that may cause the difference between the results of my study and theirs could be the hypotonicity difference, time course, and sample preparation.

Intracellular K^+ is vital to the cell. In sarcoma-180 cells, loss of intracellular K^+ has been found to be associated with a parallel depression of the rate of synthesis of protein and DNA (Lubin, 1967). In cultured rat liver slices, certain hepatotoxic agents which induce decrease in slice K^+ content also caused a depression in protein synthesis (Smith, Fisher, Shubat, Gandolfi, Krumdieck, and Brendel, 1987).

In cortical slices from rabbit, rat, and guinea pig kidneys, some studies have shown that cells swelled when exposed to hypotonic medium, lost some Na^+ and Cl^-, and reached a stable, somewhat elevated, but not grossly swollen volume that was dependent on metabolic energy but not affected by ouabain. However, loss of cellular K^+ was not found in any of these species (Hughes and Macknight, 1976).

This ouabain-insensitive regulation of cellular volume in hypotonic conditions suggests an energy-dependent volume-regulating mechanism other than the cardiac glycoside-sensitive Na^+/K^+-ATPase. Although renal cortical slices exposed to medium of half the normal osmolarity maintain their cellular K^+ content in spite of some swelling, incubation of tissues in more diluted oxygenated medium results not only in gross cellular swelling but marked loss of K^+ and in inhibition of oxygen consumption. Thus this loss of K^+ is at least partly a consequence of the swelling itself and it therefore seems wrong to regard such K^+ loss from cells incubated in very dilute media as in any way a reflection of a cellular homeostatic mechanism regulating, or attempting to regulate, cellular volume (Macknight and Leaf, 1977).

Taken together, mouse hepatocyte V_m hyperpolarizes and intracellular K^+ activity decreases during short-term hypotonic stress. This change in a^i_K can be completely explained by the cell swelling. The lack of ouabain's effect on the changes of both V_m and a^i_K induced by hypotonic shock suggests the independence of hepatocyte RVD with the Na^+/K^+ pump activity. The disagreement in cell water volumes changes between measured value and those expected (assuming hepatocytes behave as perfect osmometers) suggests that a cell volume control mechanism exists in hepatocyte during hypotonic stress. This study may suggest that an

increase in hepatocyte K^+ conductance is not necessarily accompanied by the net loss of intracellular K^+ as in hepatocytes. However, the increased K^+ conductance is necessary for setting up a more negative V_m which may be more important for hepatocyte intracellular metabolism and RVD process than simple loss intracellular K^+. Hepatocyte intracellular Cl^- has been shown to be passively distributed with V_m. Depleting hepatocyte from Cl^- impair the RVD process. So, one of the crucial parts of RVD in hepatocytes may be the change of V_m caused by increased K^+ conductance.

Hepatocyte Intracellular a^i_K Acts As a Marker of Intracellular Water Volume Change During Hyposmotic Stress

Our studies have suggested that hepatocyte total intracellular K^+ content does not decrease during short-term hypotonic stress. If this is true, then changes intracellular a^i_K should be useful as an indicator for the cellular water volume change during hypotonic stress. To test this hypothesis, hepatocyte intracellular water volume changes induced by hypotonic stress were determined by both TMA^+ method and by testing intracellular K^+ as the marker respectively. The results from paired experiments indicated that hyposmotic stress induced increase in intracellular water volume measured by TMA^+ loading technique is identical to that measured when using intracellular K^+ as the marker (Table 9). In the other words, hypotonic stress causes

equal percentage of changes in both a^i_K and intracellular TMA^+ activity. Thus my result strongly suggests that hepatocyte intracellular K^+ content remains constant during short-term hypotonic shock and its concentration change represents the intracellular water volume change in hepatocytes.

CHAPTER 6

GENERAL DISCUSSION

The Evaluation of TMA^+ Loading Technic in Determination of Hepatocytes intracellular Water Volume Changes

The technique of using intracellular loaded TMA^+ as the probe to evaluate cellular water volume change was developed by Luis Reuss in 1985 (Reuss, 1985). Pretreatment of plasma membrane with nystatin was required to load the cells with TMA^+. After withdrawal of nystatin the TMA^+ was trapped within the cells. A K^+-sensitive microelectrode was then used to measure changes in $[TMA^+]_i$. This measurement is possible, despite the high levels of $[K^+]_i$, because the resin exchanger is approximately three orders of magnitude more sensitive to TMA^+ than to K^+ (Neher and Lux, 1973). Thus, small amount of $[TMA^+]_i$ provide an impermeant intracellular volume marker and the changes in $[TMA^+]_i$ signal can be used to monitor the changes in cell water. The validation of this technique has been proven by showing that intracellular TMA^+ loading does not significantly influence the electrical properties of Necturus gallbladder epithelial cells. Their result suggests that the sensitivity of this technique permits measurement of cell volume changes of 5% or less (Reuss, 1985). In 1990, Khalbuss and Wondergem were the first to use the same technique for measuring cell water volume change in

hepatocytes (Khalbuss, and Wondergem, 1990). Their data indicates that TMA^+ loading procedure had no permanent effects on either cell viability or on the plasma membrane's relative ion permeabilities as indicated by the V_m and its increase with hyposmotic stress (Khalbuss and Wondergem, 1990). My present experiment, however, shows that pretreatment of hepatocytes with nystatin causes a significant decrease in the intracellular K^+ content although cell V_m remains constant (data not shown). This means that nystatin treatment causes cell damage and much caution should be taken when using V_m as an indicator for the viability of the cell. In order to avoid cell damage caused by nystatin, the pretreatment of nystatin is exempted from my experimental procedure. By direct exposure of hepatocytes to a perfusate where 100 mM sucrose is replaced by 50 mM TMA^+ chloride, certain amount of TMA^+ still can be transported into the cell. I attribute this to the constituent, membrane organic cation transporter in the hepatocyte plasma membrane (Moseley, Morrissette, and Johnson, 1990). In a series of preliminary experiments (data not shown), I observed that removal of TMA^+ from the extracellular bath resulted a slow dissipation of the $[TMA^+]_i$ signal, which was prevented by 5 mM TMA^+ present in the perfusate after the loading phase. After a brief equilibration period (about 20 min), the TMA^+ signal remained constant for more than 10 min in many cell

impalements. Consequently, all control and experimental media after the TMA^+-loading phase contained 5 mM TMA^+. This procedure has been validated by Adorante and Miller (1990) in retinal pigment epithelium.

The presence of 5 mM TMA^+ in the solution has no effect on hepatocyte V_m (Khalbuss and Wondergem, 1990). This indicates 5 mM TMA^+ in the bath has no effect on the plasma membrane's relative ion permeabilities. However, I found hepatocyte membrane V_m hyperpolarizes (about 6 mV) right after the cells are exposed to loading solution (data not shown). Although I assume that hepatocyte V_m will recover a little bit after removal of the loading solution for a prolonged period of time, loading hepatocytes with TMA^+ itself definitely has some effect on both resting V_m and hypotonic shock induced change in V_m (Table 9). This observation might weaken the validity of using this technique for intracellular water volume measurements in hepatocytes, especially in the case when the loaded intracellular TMA^+ concentration is relatively high. The mechanism for this effect on V_m is unclear. One possibility could be that loading hepatocyte with TMA^+ itself causes cell swelling. This could stimulates the swelling activated K^+ channels on the cell membrane. Since the cell has already swollen to certain degree during intracellular TMA^+ loading, the hypotonic shock applied afterward then stimulates a mechanism that has been activated by prior cell

swelling. This can explain the higher resting V_m and the lower percentage of change in V_m induced by hypotonic stress in TMA^+ group of Table 9. If the above explanation is true, then the percentage changes of intracellular water volume measured in my experiments could be underestimated. However, I don't have evidence for that because almost all of my data agree well with those reported by others. The mechanisms for TMA^+ uptake into hepatocytes during the process of TMA^+ loading is probably through the non-specific organic cation transporter on the hepatocyte plasma membrane. In retinal pigment epithelium of Bullfrog (Rana catesbeiana). Adorante et al have examined a variety of cation transport mechanisms known to locate at retinal pigment epithelium apical membrane. None of these transporters are responsible for net TMA^+ uptake. They proposed that the net TMA^+ uptake is through a yet unspecified organic cation pathway (Adorante and Miller. 1990). Another possibility is that the TMA^+ is transported into hepatocytes through the organic cation-proton exchanger in membrane. This organic cation-proton antiport has been demonstrated in the sinusoidal membrane of rat liver (Richard, M., J. Morrissette, and T. R. Johnson. 1990). The activation of this antiport causes the intracellular accumulation of organic cation and the increase in the intracellular pH. Since the V_m has been shown to be hyperpolarized by increase in intracellular pH, this could

explain the TMA^+ loading induced increase in V_m in my experiments.

The General Model of Hepatocyte Volume Response During Hyposmotic Stress and L-alanine Uptake in Mouse Liver Slices

Based on my present study and the studies of others, the response of hepatocytes in liver slices to hypotonic shock is similar to that during L-alanine uptake. But it is likely that the two volume control mechanisms are not exactly the same because the later involves an increase in intracellular Na^+ content and, thus, an activation of the Na^+/K^+ pump. Na^+/K^+ pump activity remains unchanged during RVD in hypotonically perfused rat liver (Haddad and Graf, 1989).

The proposed volume control mechanism during hypotonic stress is as follows: Hypotonic shock causes hepatocytes to swell. Cell swelling stimulates the increase in both K^+ and Cl^- conductance of the plasma membrane. This process might be mediated by an increased intracellular free Ca^{2+} or other secondary messengers within the cell. The increased K^+ conductance induces a hyperpolarization of the V_m, although no detectable net loss of intracellular K^+ could be found. The more electrically negative intracellular environment provides an electromotive force for the efflux of intracellular Cl^- (accompanied with cations), which in turn contributes to the cell volume recovery osmotically.

In the case of L-alanine uptake, the proposed volume control mechanism is as follows: Hepatic alanine uptake through the Na^+-alanine cotransport systems results in an increase in hepatocyte intracellular Na^+ content. The increased $[Na^+]_i$ stimulates the Na^+/K^+ pump activity. Meanwhile, water moves in due to the increased concentration of the intracellular osmotically active particles. This causes cell swelling which in turn stimulates the increases of K^+ and Cl^- conductance of the plasma membrane. Both activation of the Na^+/K^+ pump and the increase in K^+ conductance induce a hyperpolarization of the V_m. The hyperpolarized V_m provides the electromotive force for the efflux of Cl^- which then prevents hepatocytes from further swelling. The cations accompanying the efflux of Cl^- are unclear. Na^+ might be the candidate (Russo, van Rossum, and Galeotti, 1977). A cartoon of L-alanine induced RVD in mouse hepatocytes as mentioned above is shown in Fig. 18.

Possible Second Messengers and Metabolic Alterations Involved in Hepatocyte Regulatory Volume Decrease

Relatively little is known about the secondary messengers involved in the volume regulatory mechanisms in hepatocytes. But it may be interest to note that full activation of the volume-regulatory response, at least as pertains to K^+ flux, comes about with a certain delay, possibly indicating that intermediate steps exist between

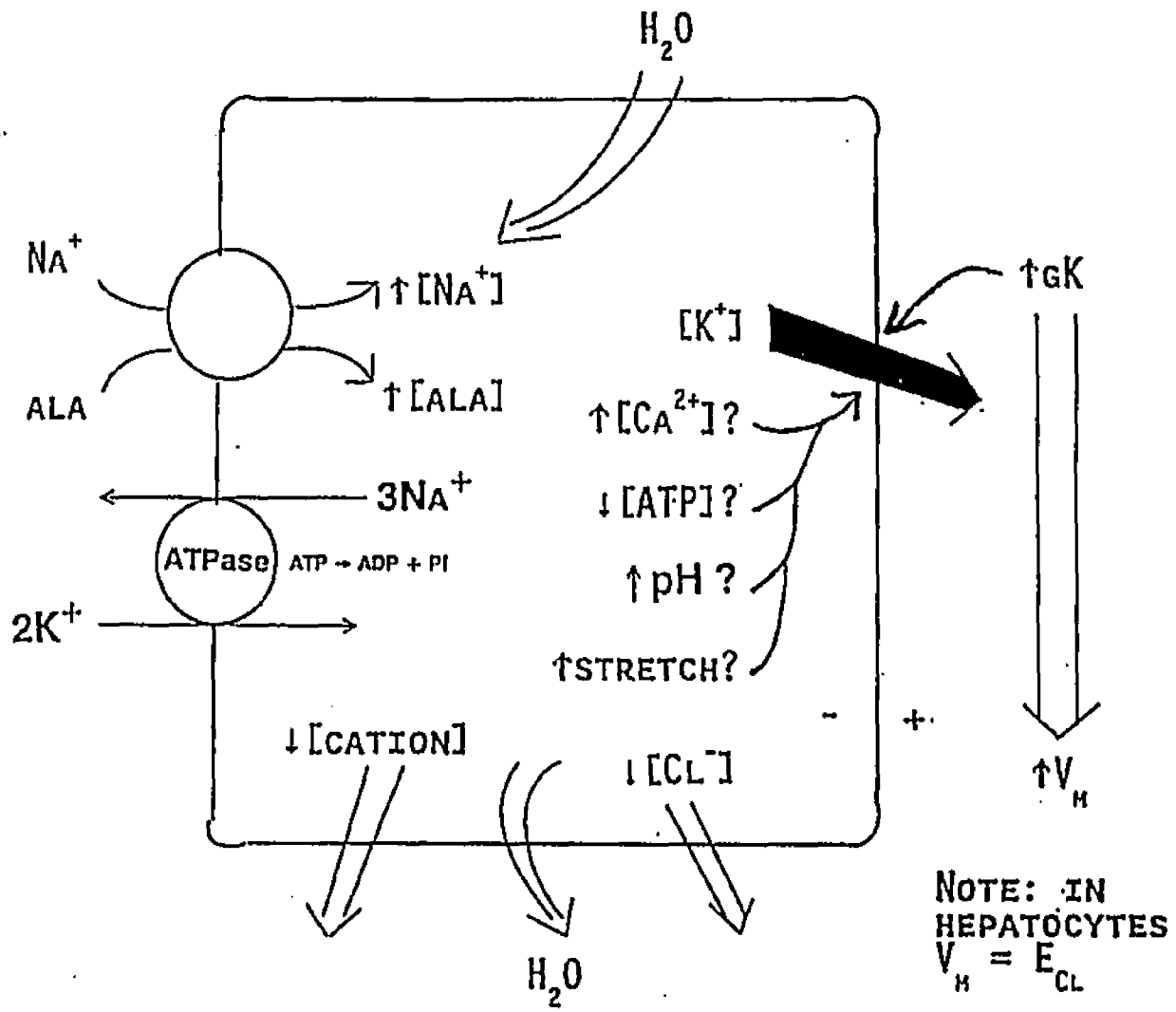

Fig. 18. The proposed model for hepatocyte regulatory volume decrease during L-alanine uptake.

the volume-sensing mechanisms and the modulation of volume-regulatory ion fluxes (Graf, Haddad, Haeussinger, and Lang, 1988). Hypotonic cell swelling modifies intracellular signalling systems, such as Ca^{2+}, intracellular pH, inositol phospholipids and eicosanoids, hyperpolarizes the membrane potential and changes the ionic composition inside the cell (Hallbrucker, vom Dahl, Lang, and Haussinger, 1991). Bear (1990) reported the presence of Ca^{2+} activated K^+ channel in the plasma membrane of rat hepatocytes and a hypotonic shock induced elevation in cytosolic Ca^{2+}. This change in intracellular Ca^{2+} concentration was dependent on extracellular Ca^+ concentration. She also found the hypotonic shock-induced RVD was slightly but significantly inhibited by depletion of extracellular Ca^{2+}. Her studies suggest that stretch-activated nonselective cation channels in liver cells permit the transient influx of Ca^{2+}, which in turn acts to trigger changes in ion conductance or cytoskeletal components involved in cell volume regulation (Bear, 1990; Bear and Petersen, 1987). Khalbuss and Wondergem reported that both extracellular and intracellular Ca^{2+}, calmodulin

system, and microfilaments are involved in control and activation of hypotonic stress-induced increase in hepatocyte V_m. This change in hepatocyte V_m plays a role in cell volume regulation. (Khalbuss and Wondergem, 1991).

Cell volume regulation not only depends on ion

transport but may involve metabolic processes which create or degrade organic osmolytes, such as amino acids, methylamines, and polyols. A role for organic osmolytes during cell volume regulation has been established for a variety of nonmammalian species, for Ehrlich ascites tumor cells, for brain cells, and for kidney medulla. Very little is known about metabolic processes influenced by cell swelling or shrinkage in liver cells. However, a few observations suggest the alterations of liver cell metabolism during disturbances of cell volume (Graf, Haddad, Haeussinger, and Lang, 1988). Both hypotonic- and amino acid-induced cell swelling has been found to inhibit proteolysis in perfused rat liver (Haussinger, Hallbrucker, vom Dahl, Lang, and Gerok, 1990; Hallbrucker, vom Dahl, Lang, and Haussinger, 1991; Wettstein, vom Dahl, Lang, Gerof, and Haussinger, 1990). It has been shown that hypotonic perfusate leads to a reduced oxygen uptake and accordingly reduced oxidative metabolism and a reduced hydrogen ion release during liver cell swelling. Lactate, pyruvate and glucose release are reduced during exposure of liver cells to hypotonic perfusate and they are enhanced following exposure to hypertonic perfuastes. In perfused liver, exposure to hypotonic perfusate depresses and exposure to hypertonic perfusate stimulates glycogenolysis and glycolysis. The alterations of substrate release clearly outlast the rapid movements of K^+, indicating that

the cell remains in a different metabolic state after seeming completion of rapid cell volume regulation. The alteration of metabolism may contribute to the slow adjustment of cell volume (Graf, Haddad, Haeussinger, and Lang, 1988).

CHAPTER 7

SUMMARY, CONCLUSION, AND PROSPECTIVE

In summary, hepatocyte volume regulatory mechanisms are much more complicated than expected. It is likely that these volume regulatory processes are, at least, composed of the following components: 1) the alterations of membrane ionic permeabilities, the activities of the ionic transporters on the plasma membrane and accordingly the ionic fluxes across the membrane; 2) the changes of intracellular Ca^{2+} activity and other secondary messenger systems; 3) the alteration of intracellular metabolism; 4) the changes in the rate of bile secretion and microtubule mediated exocytosis and finally 5) a change in the tension and structural orientation of the cytoskeleton system.

My study has been focused mainly on the first part and may represent the initial cellular response to the disturbance of the osmotic balance across the cell membrane. The major discoveries of my present study are: 1) It directly demonstrates the correlation between the osmotic stress induced changes of hepatocyte V_m and the intracellular Cl^- activity. Osmotic stress induced change of V_m provides electromotive forces for the fluxes of Cl^- across cell membrane. This change in a^i_{Cl} could be crucial for hepatocyte volume regulation. 2) It directly demonstrates the decrease of hepatocyte intracellular K^+

activity during short-term hypotonic shock. This decrease of a^i_K is attributed mainly to the cell swelling, although other K^+ transport mechanisms might be involved. In the other words, it appears that hepatocytes would not lose their intracellular K^+ when challenged with hypotonic stress, especially in the physiological range. 3) My study provides direct, continuous, simultaneous recordings showing the relevant changes of hepatocyte V_m and the intracellular Cl^- activity induced by L-alanine uptake. 4) My study also provides evidence suggesting differences between the hypotonic stress-induced RVD and that induced by amino acid uptake in mouse hepatocytes. The activity of the Na^+/K^+ pump differs between these two conditions based on the differences in the intracellular Na^+ concentration.

Normal volume maintenance in hepatocytes is a constant energy-consuming process. Inhibition of cellular metabolism causes hepatocytes swell in isotonic media with physiological ion concentrations (Graf, Haddad, Haussinger, and Lang, 1988). In addition to this, cell volume regulation must also deal with alterations of solute concentrations either inside or outside the cell. These may results from a variety of factors such as intracellular polymerization and depolymerization processes (e.g. synthesis and degradation of proteins or glycogen), changes in the concentration of intermediates of cell metabolism, variations of nutrient or salt concentrations in the portal

blood (Graf, Haddad, Haussinger, and Lang, 1988). Recent studies have indicated that amino acids, even in physiological concentrations, elicit liver cell swelling and volume-regulatory ion-flux responses (Wettstein, vom Dahl, Lang, Gereof, and Haussinger, 1990; Hallbrucker, vom Dahl, Lang, and Haussinger, 1991). From the view of pathology, two of the earliest and most conspicuous features of hepatic damage induced by alcohol are the deposition of fat and enlargement of the liver (Lieber, 1991). This is due in part (up to 25%) to an increase in cellular lipids, but most of the increase in cell volume is due to accumulation of cell water secondary to an increase in cell K^+ and protein content, which behave as osmolytes (Israel, Orrego, Colman, and Britton, 1982; Baraona, Borowsky, and Lieber, 1975). The consequence of increase in the volume of hepatocytes is smaller sinusoids, which leads to portal hypertension, impedes blood flow, and results in functional hypoxia (Israel and Orrego, 1987). The mechanisms by which the chronic alcohol ingestion leads to hepatocyte swelling and the sequelae of degenerative disease is not quite clear. However, ethanol induced increase in cellular protein content could be the result of acetaldehyde-induced impairment of microtubular mediated protein secretion. Acetaldehyde is the intermediate metabolite of ethanol oxidation in hepatocytes and this highly-reactive metabolite binds to various hepatic proteins including tubulin.

Ethanol has also been found to increase the fluidity of the plasma membrane, alter glycoprotein and certain enzymes in the plasma membrane of liver cells (Lieber, 1991). An ethanol induced transient increase in hepatocyte cytosolic free Ca^{2+} has been reported in isolated hepatocytes (Hoek, Thomas, Rubin, and Rubin, 1987). Thus, it is possible that one of the mechanisms for ethanol induced liver injury is due to its disrupting effect on hepatocyte volume controlling systems. Change of hepatocyte volume could also influence the normal functions of other organs such as kidney. Glutamine induced liver cell swelling has been shown to lead marked decreases in renal glomerular filtration rate, renal para-aminohippurate clearance and urinary flow rate. This could be the result of stimulating effect of increased intrahepatic pressure on the renal nerve activity induced by liver cell swelling (Lang, Tschernko, Schulze, Ottle, Ritter, Volkl, Hallbrucker, and Haussinger, 1991).

Studies on the mechanisms of hepatocyte volume regulation are still in their early stage. More work needs to be done in the aspects of the secondary messenger systems, intracellular metabolic process, microtubule mediated exocytosis, bile secretion, and the role of the cytoskeleton system during hepatocyte volume regulation.

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